

## SUPPLEMENTARY INFORMATION

### **Structural basis of Nipah virus RNA synthesis**

**Fernanda A. Sala<sup>1,2</sup>, Katja Ditter<sup>1,2</sup>, Olexandr Dybkov<sup>3</sup>, Henning Urlaub<sup>3,4,5</sup> and Hauke S. Hillen<sup>1,2,5,6\*</sup>**

<sup>1</sup>Department of Cellular Biochemistry, University Medical Center Göttingen, Göttingen, Germany

<sup>2</sup>Research Group Structure and Function of Molecular Machines, Max Planck Institute for Multidisciplinary Sciences, Göttingen, Germany

<sup>3</sup>Bioanalytical Mass Spectrometry Group, Max Planck Institute for Multidisciplinary Sciences, Göttingen, Germany

<sup>4</sup>Bioanalytics Group, University Medical Center Göttingen, Institute for Clinical Chemistry, Göttingen, Germany

<sup>5</sup>Cluster of Excellence “Multiscale Bioimaging: from Molecular Machines to Networks of Excitable Cells” (MBExC), University of Göttingen, D-37075 Göttingen, Germany

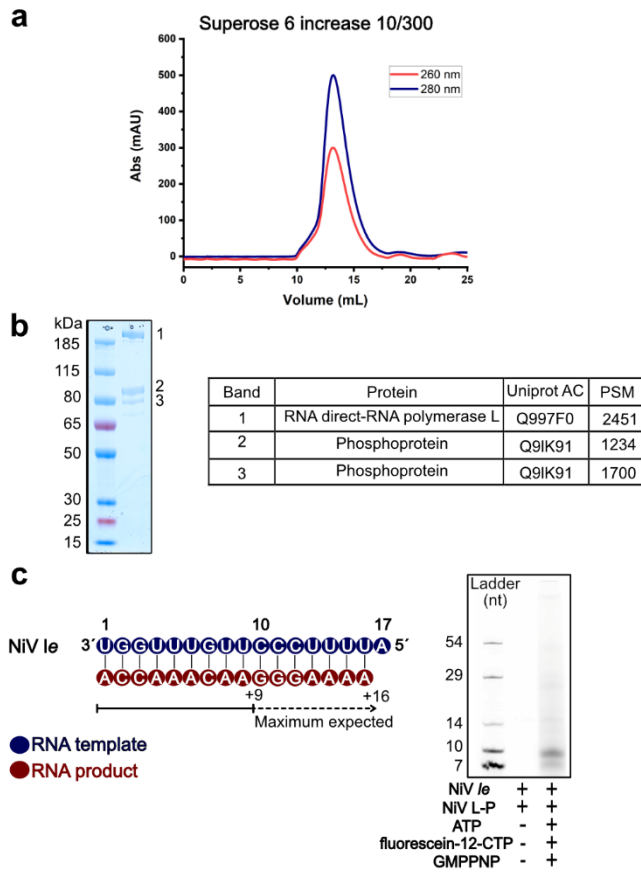
<sup>6</sup>Göttingen Center for Molecular Biosciences (GZMB), Research Group Structure and Function of Molecular Machines, University of Göttingen, D-37077 Göttingen, Germany

\*Corresponding author. E-mail: [hauke.hillen@med.uni-goettingen.de](mailto:hauke.hillen@med.uni-goettingen.de)

#### **This PDF file includes:**

Figures S1 to S8

Table S1

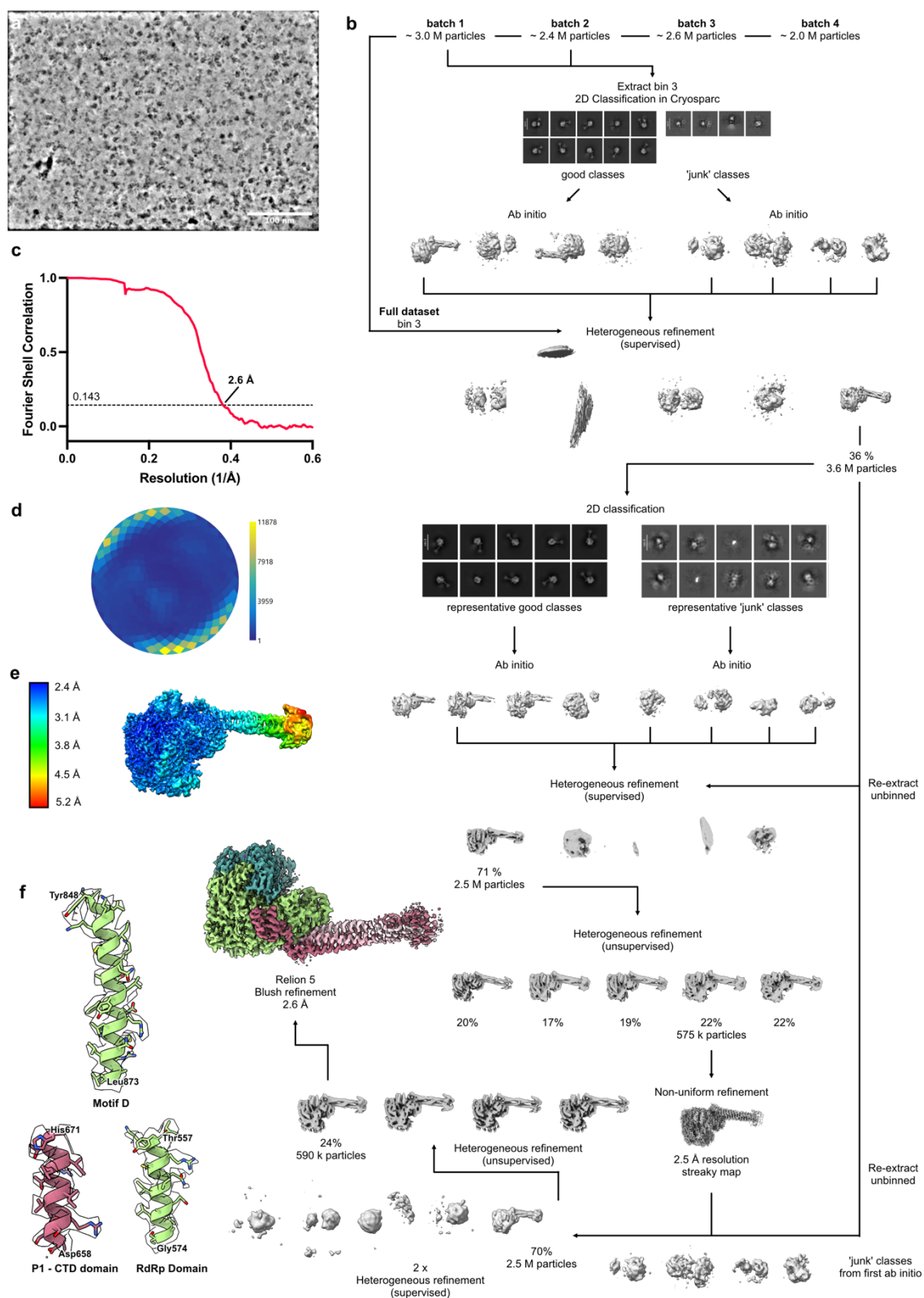


### Supplementary Fig. 1: Purification and activity of recombinant NiV L-P complex.

a) Size exclusion chromatography profile of the NiV L-P complex using a Supersrose 6 Increase 10/300 GL column. The absorbance at 260 nm and 280 nm is shown in red and blue, respectively. Source data are provided as a Source Data file.

b) SDS-Page analysis of the purified NiV L-P complex using a 4-12% polyacrylamide gel. Proteins in visible bands were identified by mass spectrometry. The top hit for each band and respective number of peptide-spectrum matches (PSM) are shown in a table. Due to a lower intensity of band 3, 3 times more of this sample was injected into the mass spectrometer as compared to the bands 1 and 2.

c) RNA synthesis assay using fluorescein-labeled CTP. Products were separated on a 20% urea gel and visualized using with a Typhoon phosphorimager (GE Healthcare). RNA product lengths were determined by comparing them to a ladder of synthetic RNA molecules of known sizes. The experiment was performed in triplicate. Source data are provided as a Source Data file.



**Supplementary Fig. 2: Cryo-EM processing workflow for the apo NiV L-P complex.**

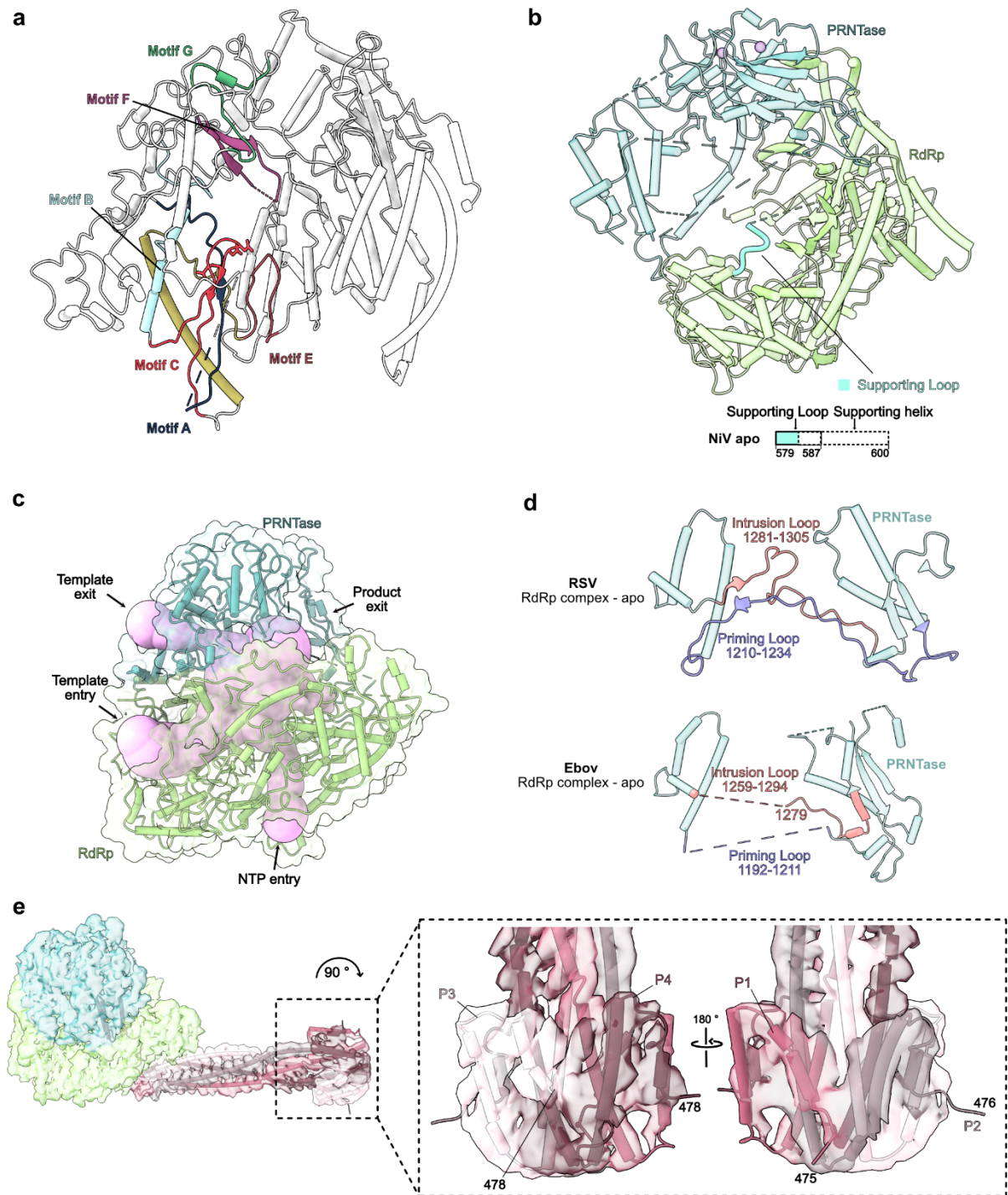
a) Representative denoised micrograph.

b) Schematic of image processing workflow. See Methods for details.

c) FSC plot of final reconstruction.

- d) Angular distribution plot created with Warp<sup>48</sup>.
- e) Local resolution filtered map (created in Relion 5.0<sup>50</sup>) colored by local resolution.
- f) Densities and atomic models for representative structural elements.





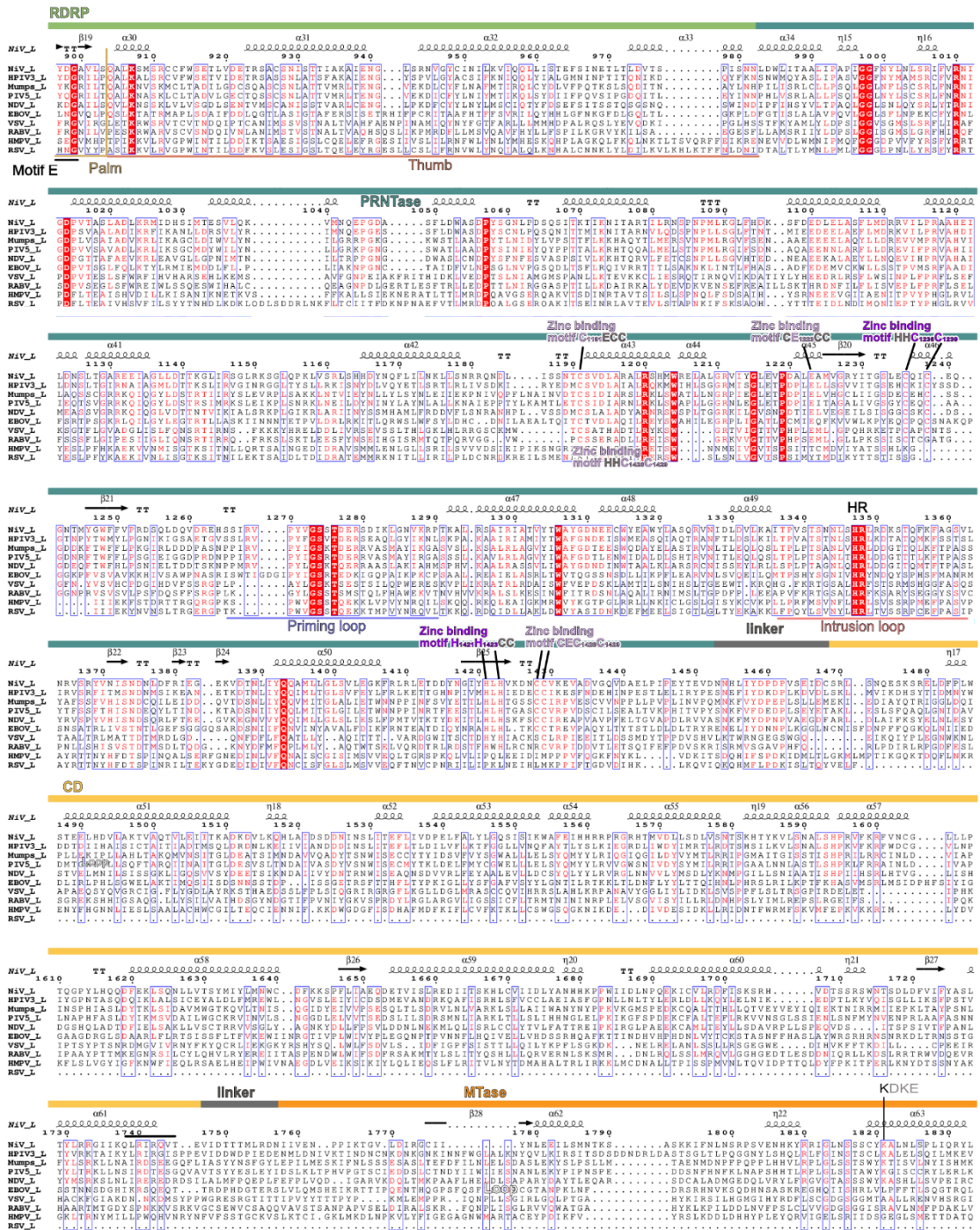
**Supplementary Fig. 3: Details and comparison of apo NiV L-P complex.**

a) Cartoon depiction of conserved catalytic motifs. The RdRp domain is color-coded with catalytic motifs from A to G displayed and labeled in distinct colors.

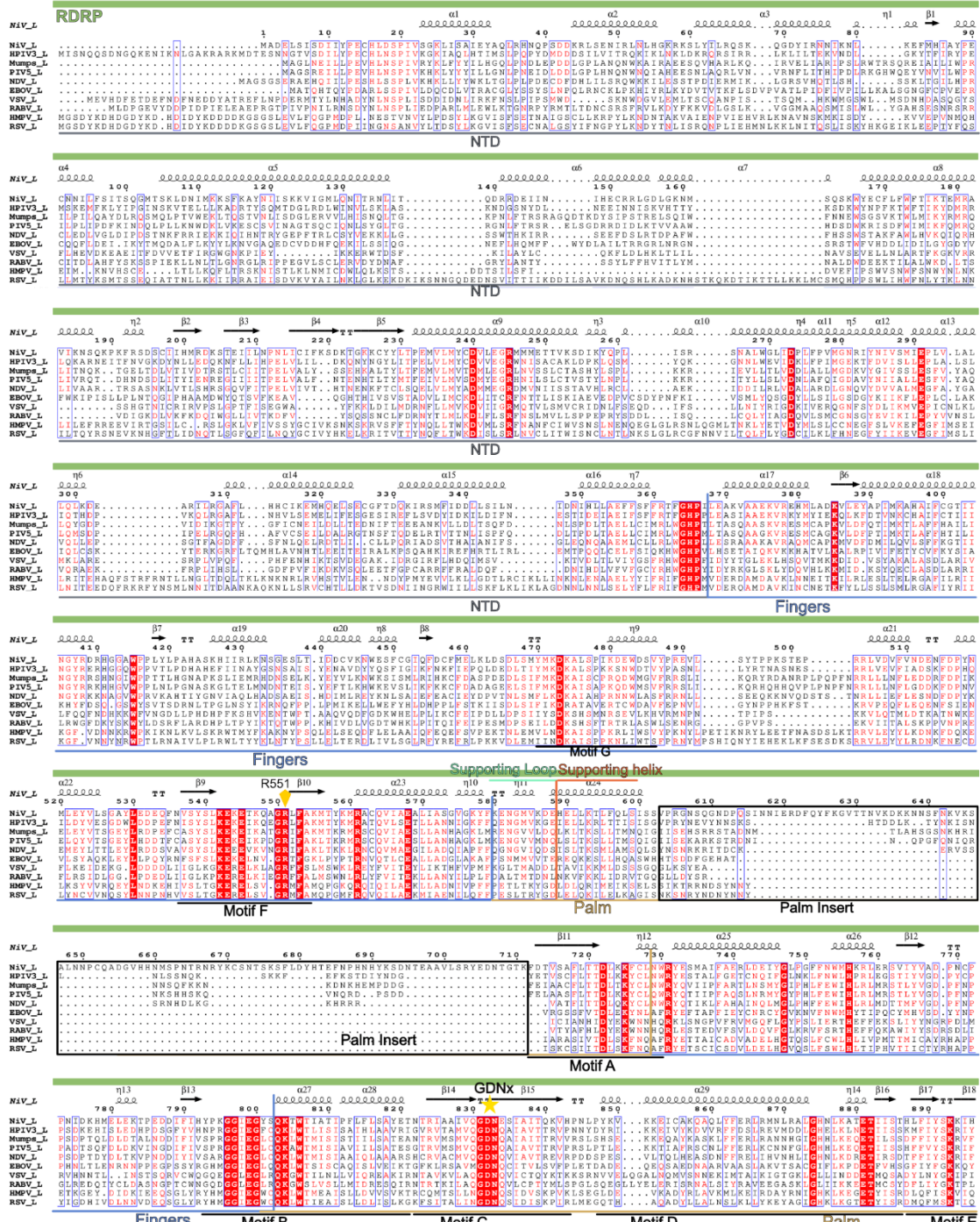
b) RdRp and PRNTase domains are shown as cartoon with structural and schematic depiction of the supporting helix and loop.

c) Presumable RNA tunnels within the NiV L-P complex. Cavities in the complex were calculated using CAVERweb<sup>60</sup>[Click or tap here to enter text.](#) and are represented by pink spheres.

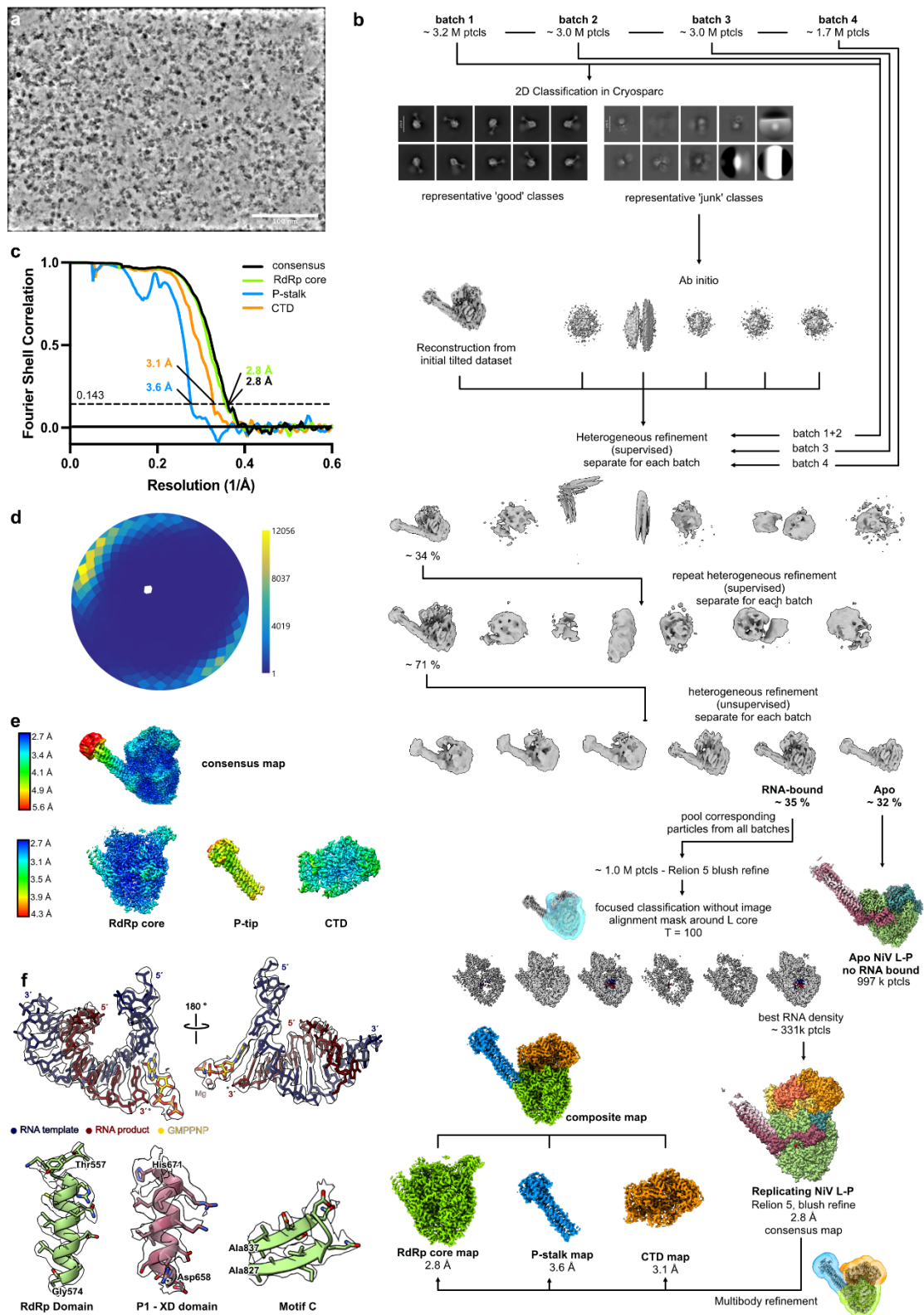
d) Comparison of the priming and intrusions loops of in apo NiV L-P complex with the apo RSV L-P complex (PDB ID: 6UEN)<sup>27</sup> and apo EBOV L-P complex (PDB ID: 7YER)<sup>10</sup>. e) Detailed view of the unique P-stalk tip of the NiV L-P complex. The apo NiV L-P complex structure is displayed as cartoon with the local resolution filtered cryo-EM map as transparent surface.











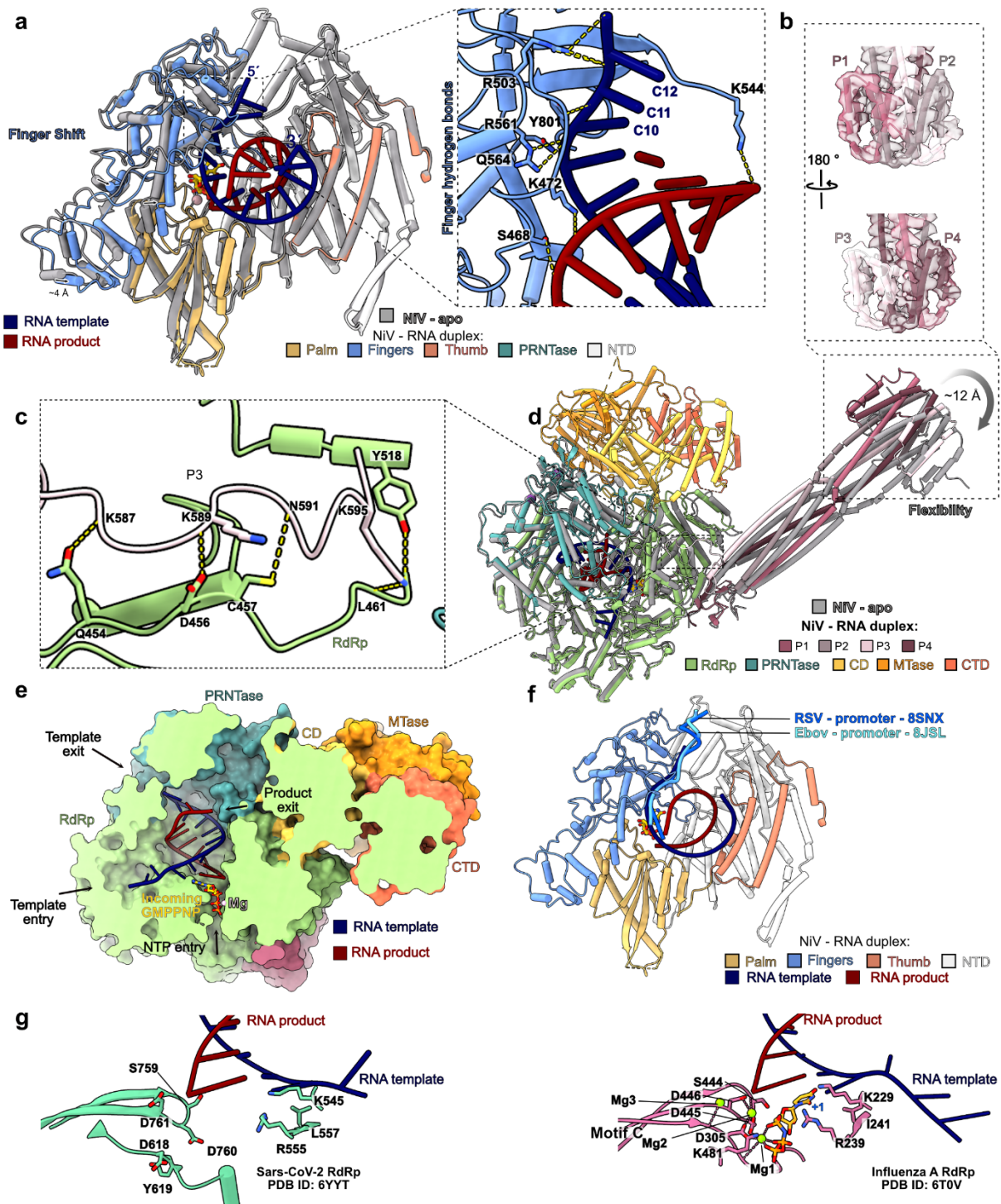
**Supplementary Fig. 5: Cryo-EM processing workflow for the elongating NiV L-P complex.**

a) Representative denoised micrograph.

b) Schematic of image processing workflow. See Methods for details.

- c) FSC plot of final reconstruction.
- d) Angular distribution plot created with Warp<sup>48</sup>.
- e) Local resolution filtered maps (created in Relion 5.0<sup>50</sup>) colored by local resolution.
- f) Composite cryo-EM density map and atomic models for ligands and representative structural elements.



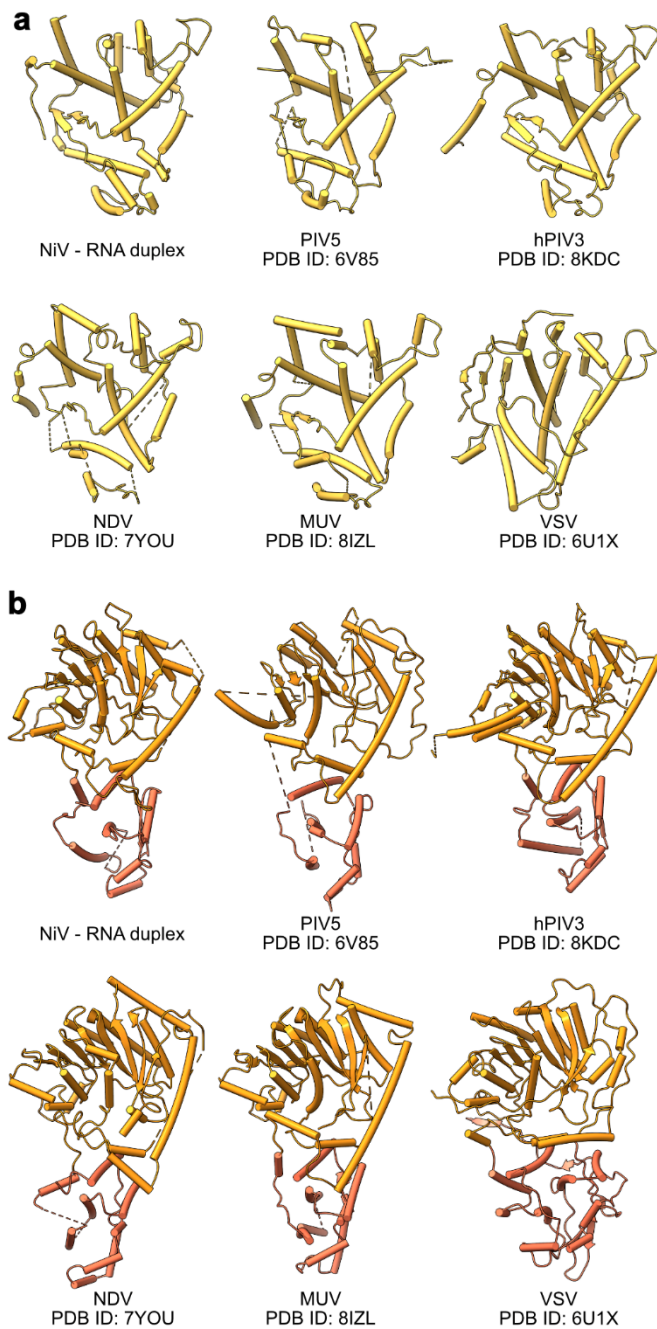


**Supplementary Fig. 6: Details and comparison of elongating NiV L-P complex.**

a) Superimposition of the NiV L RdRp domain in apo (gray) and elongating state (color-coded: finger domain in blue, thumb in salmon, palm in golden yellow, and NTD in gray). The active site and GMPPNP are shown as sticks, with  $Mg^{2+}$  ions as pink spheres. The inset displays the interactions between the finger domain and the RNA template, with hydrogen bonds indicated by dashed lines.



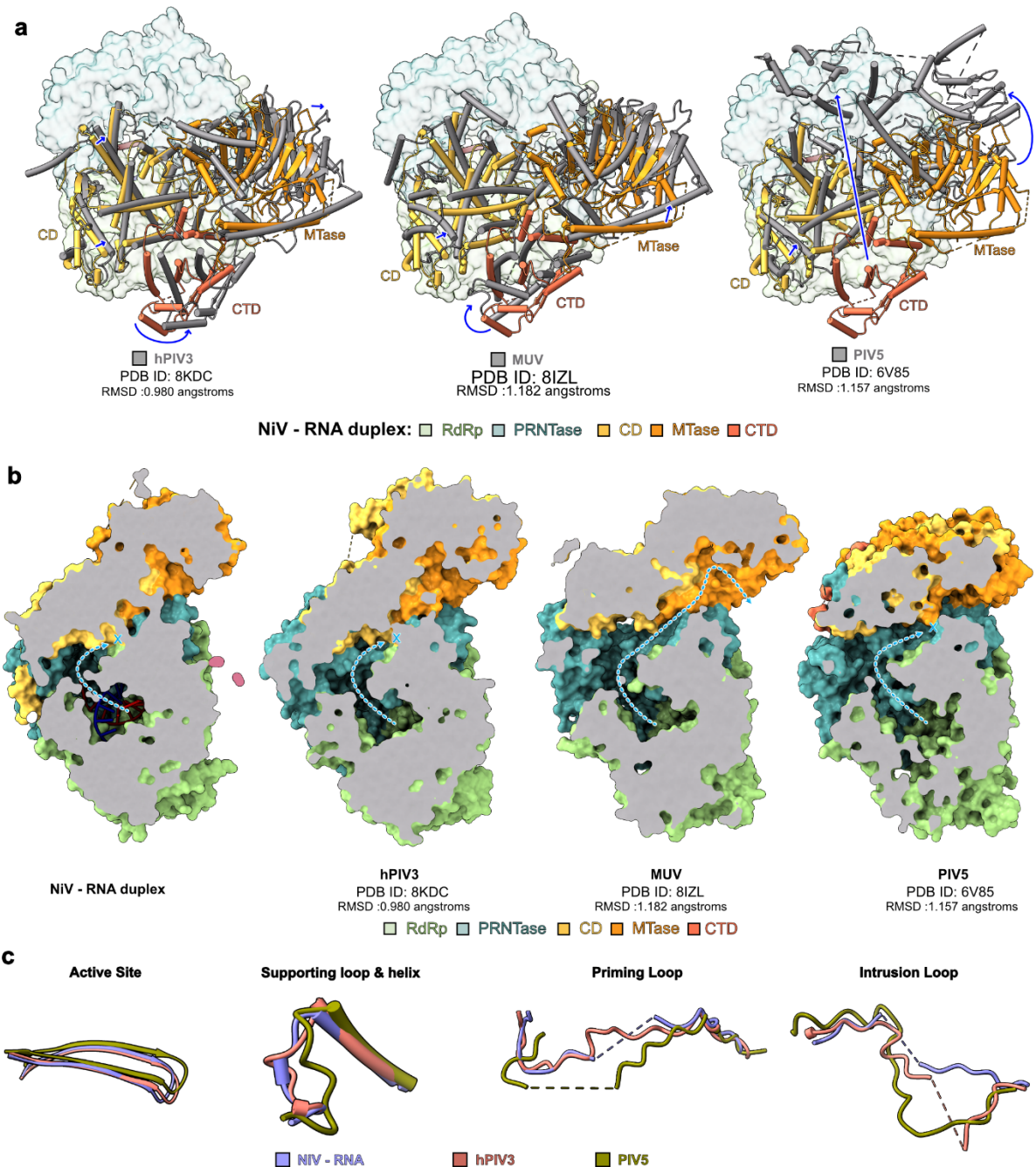
- b) Close-up view of the P-stalk tip of elongating NiV L-P complex. The apo NiV L-P complex structure is displayed as cartoon with the composite cryo-EM map as transparent surface.
- c) Close-up view of the interactions between P3 and the L RdRp domain.
- d) Superimposition of apo (gray) and elongating (colored) NiV L-P complex structures shows conformational flexibility of the P-stalk.
- e) Slice-through view of elongating NiV L-P complex. The structure is displayed as surface representation with a partially clipped surface. The RNA template and GMPPNP are shown as cartoons and stick, respectively. The template entrance and exit, the NTP entrance and product exit are highlighted
- f) Comparison to promoter-bound nsNSV L-P structures. The RdRp domain of elongating NiV L is colored as in panel a. The structures of EBOV (PDB ID: 8JSL)<sup>16</sup> and RSV L-P complex (PDB ID: 8SNX)<sup>17</sup> in complex with promoter RNA were superimposed and the RNA is shown as ribbon. The template RNA follows a similar trajectory in all complexes.
- g) Conserved active site architecture across different RNA virus polymerases. (Left) Close-up view of the SARS-CoV-2 RdRp active site (PDB ID: 6YYT)<sup>34</sup>. (Right) Close-up view of the Bat-Influenza A RdRp active site (PDB ID: 6T0V)<sup>35</sup>. Residues interacting with RNA are shown as sticks. The incoming NTP is depicted as yellow sticks and Mg<sup>2+</sup> ion as green spheres.



**Supplementary Fig. 7: Structural comparison of CD and MTase from NiV and related viruses.**

a) Comparison of the CD domains of Nipah virus, parainfluenza virus 5 (PIV5, PDB ID: 6V85)<sup>14</sup>, human parainfluenza virus 3 (hPIV3, PDB ID: 8KDC)<sup>13</sup>, Newcastle disease virus (NDV, PDB ID: 7YOU)<sup>18</sup>, Mumps virus (PDB ID: 8IZL)<sup>15</sup>, and vesicular stomatitis virus (VSV, PDB ID: 6U1X)<sup>19</sup>.

b) Comparison of the methyltransferase (MTase) domain (colored in gold yellow) and CTD (orange) of the viruses described in (a)



**Supplementary Fig. 8: Structural comparison of L flexible domains from NiV and related viruses.**

a) Superimposition of the L RdRp domain of elongating NiV L-P complex with hPIV3 (left, PDB ID: 8KDC, RMSD: 0.980 Å)<sup>13</sup>, MUV (middle, PDB ID: 8IZL, RMSD: 1.182 Å)<sup>15</sup> and PIV5 (right, PDB ID: 6V85, RMSD: 1.157 Å)<sup>14</sup>. The RdRp and PRNTase domains are displayed as surfaces in light green and dark cyan, respectively. The CD, MTase, and CTD regions are shown as cartoons, colored as in Fig. 2a for elongating NiV L-P complex and in gray for the other structures.

- b) Slice-through views of elongating L-P complexes of nsNSVs, aligned as in panel a). The structures are shown in surface representation with a partially clipped surface, highlighting the RNA product exit tunnel.
- c) Comparison of active site motif C, supporting loop and helix, priming loop, and intrusion loop between NiV, hPIV3 (PDB ID: 8KDC)<sup>13</sup>, and PIV5 (PDB ID: 6V85)<sup>14</sup> L-P complexes. Color-coding as indicated.

## SUPPLEMENTARY TABLE

**Supplementary Table 1 | Cryo-EM data collection, refinement and validation statistics**

	<b>Apo Nipah L-P complex PDB: 9GJT EMD-51402</b>	<b>Elongating Nipah L-P complex PDB: 9GJU EMD-51403</b>
<b>Data collection and processing</b>		
Magnification	105,000 x	105,000 x
Voltage (kV)	300	300
Electron exposure (e-/Å <sup>2</sup> )	48.94	52.00
Defocus range (µm)	0.5 – 2.5	0.5 – 2.5
Pixel size (Å)	0.834	0.834
Symmetry imposed	C1	C1
Initial particle images (no.)	9,886,170	10,957,464
Final particle images (no.)	591,312	330,750
Map resolution (Å)	2.6	2.8
FSC threshold	0.143	0.143
Map resolution range (Å)	2.4 – 5.2	2.7 – 5.6
Map sharpening <i>B</i> factor (Å <sup>2</sup> )	-93	-47
<b>Refinement</b>		
Model resolution (Å)	2.7	2.8
FSC threshold	0.5	0.5
Model composition		
Non-hydrogen atoms	14,017	20,948
Protein residues	1,741	2,540
Ligands	2 x ZN	2 x ZN, 1 x MG, 1 x GMPPNP
<i>B</i> factors (Å <sup>2</sup> )		
Protein	81.5	54.6
Ligand	96.8	21.7
R.m.s. deviations		
Bond lengths (Å)	0.003	0.002
Bond angles (°)	0.541	0.481
Validation		
MolProbity score	1.19	1.32
Clashscore	3.99	5.74
Poor rotamers (%)	0.44	1.03
Ramachandran plot		
Favored (%)	98.02	98.41
Allowed (%)	1.92	1.48
Disallowed (%)	0.06	0.12

## Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our [Editorial Policies](#) and the [Editorial Policy Checklist](#).

### Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- |                                     |                          |  |
|-------------------------------------|--------------------------|--|
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | The exact sample size ( $n$ ) for each experimental group/condition, given as a discrete number and unit of measurement  |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly  |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | The statistical test(s) used AND whether they are one- or two-sided<br><i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i>   |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | A description of all covariates tested   |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons  |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | For null hypothesis testing, the test statistic (e.g. $F$ , $t$ , $r$ ) with confidence intervals, effect sizes, degrees of freedom and $P$ value noted<br><i>Give <math>P</math> values as exact values whenever suitable.</i>                            |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings   |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes   |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | Estimates of effect sizes (e.g. Cohen's $d$ , Pearson's $r$ ), indicating how they were calculated   |

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

### Software and code

Policy information about [availability of computer code](#)

Data collection Serial EM v4.0, Xcalibur 4.4

Data analysis Warp v1.0.9, Cryosparc v.4.5.3, Relion 5.0, Phenix 1.21.1-5286-000, ChimeraX 1.8-rc2024.05.16, ISOLDE 1.6.0, Coot 0.0.8.94, AlphaFold 3, MaxQuant 2.6.1.0

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

### Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The electron potential reconstructions were deposited with the Electron Microscopy Database (EMDB) under accession codes EMD-51402 [<https://www.ebi.ac.uk/pdbe/entry/emdb/EMD-51402>] (apo NIV L-P complex), and for the elongating NIV L-P complex: EMD-51403 [<https://www.ebi.ac.uk/pdbe/entry/emdb/EMD-51403>] (composite map), EMD-51723 [<https://www.ebi.ac.uk/pdbe/entry/emdb/EMD-51723>] (Map 1 - RdRp core), EMD-51724 [<https://www.ebi.ac.uk/pdbe/entry/emdb/>]

EMD-51724] (Map-2 – P protein), EMD-51725 [https://www.ebi.ac.uk/pdbe/entry/emdb/EMD-51725] (Map 3 – Large protein C-terminal) and EMD-51722 [https://www.ebi.ac.uk/pdbe/entry/emdb/EMD-51722] (consensus map). The structure coordinates were deposited to the Protein Data Bank (PDB) under accession codes 9GJT [http://doi.org/10.2210/pdb9GJT/pdb] (apo NiV L-P complex) and 9GJU [http://doi.org/10.2210/pdb9GJU/pdb] (elongating NiV L-P complex). Source data are provided with this paper.

## Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender	N/A
Reporting on race, ethnicity, or other socially relevant groupings	N/A
Population characteristics	N/A
Recruitment	N/A
Ethics oversight	N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

## Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://nature.com/documents/nr-reporting-summary-flat.pdf)

## Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No statistical methods were used to predetermine sample size. Cryo-EM data were collected on a grid prepared from one in vitro reconstituted complex. This is standard practice in Cryo-EM.
Data exclusions	No data were excluded from the analysis.
Replication	All attempts at replication were successful. Three technical replicates were carried out for the in vitro activity assay.
Randomization	The experiments were not randomized.
Blinding	The investigators were not blinded to allocation during experiments and outcome assessment.

## Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> Antibodies
<input type="checkbox"/>	<input checked="" type="checkbox"/> Eukaryotic cell lines
<input checked="" type="checkbox"/>	<input type="checkbox"/> Palaeontology and archaeology
<input checked="" type="checkbox"/>	<input type="checkbox"/> Animals and other organisms
<input checked="" type="checkbox"/>	<input type="checkbox"/> Clinical data
<input checked="" type="checkbox"/>	<input type="checkbox"/> Dual use research of concern
<input checked="" type="checkbox"/>	<input type="checkbox"/> Plants

### Methods

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> ChIP-seq
<input checked="" type="checkbox"/>	<input type="checkbox"/> Flow cytometry
<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

## Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)	Hi5 cells: Expression Systems, Tni Insect cells in ESF921 media, item 94-002F Sf9 cells: ThermoFisher, Catalogue Number 12659017, Sf9 cells in Sf-9000TM III SFM Sf21 cells: Expression Systems, SF21 insect cells in ESF921 medium, Item 94-003F
Authentication	None of the cell lines were authenticated.
Mycoplasma contamination	Cell lines were not tested for mycoplasma contamination.
Commonly misidentified lines (See <a href="#">ICLAC</a> register)	No commonly misidentified cell lines were used.

## Plants

Seed stocks	N/A
Novel plant genotypes	N/A
Authentication	N/A



# Structural basis of Nipah virus RNA synthesis

Corresponding Author: Professor Hauke Hillen

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this study, the authors resolved two high-resolution structures of the NiV L-P complex in its apo and early-elongation states. The latter one represents a structural snapshot of an actively elongating L-P complex, with template RNA, product RNA, and incoming NTP bound, which is the highlight of the study. This finding helps enrich the understanding of nsNSV L-P complex during the RNA synthesis.

Major points:

1. Replication or transcription. The key finding of this work is to resolve the structure of NiV L-P complex with template RNA, product RNA, and incoming NTP bound. The authors claimed this state as the "replication" state throughout the manuscript including the title. Is there any evidence to strongly support this claim? The biochemical assay provided in the manuscript can only be claimed as "RNA synthesis". The difference between genome replication and transcription mainly lies in the channel connectivity to the MTase and the RNA coated by nucleoprotein during replication. It will be great if authors compare the resolved structures with other nsNSV L-P complexes following the reference (Li, T. et al. Structures of the mumps virus polymerase complex via cryo-electron microscopy. Nat. Commun. 15, 4189 (2024)).
2. RNA in the apo-state NiV L-P complex. In the extended data Figure 5, authors also found the apo-state NiV L-P complex (the ratio at 32%) in the whole dataset. It is not clear whether there is RNA, no matter template RNA or product RNA, resolved in this structure. If not, RNA may play an important role in determining more density. Can authors increase the ratio of NiV L-P with the appendage (CTD map) via elevating RNA ratio?
3. Nomenclature. It is confused to use NiV L-P complex as NiV RdRp complex, since L has a RdRp domain. The same nomenclature issue occurs on P. Four P molecules in P tetramer are usually depicted as P1, P2, P3, and P4, respectively. It is better to keep consistent with other papers in the field.
4. Line 22/350: The article of "Yang, G., Wang, D. & Liu, B. Structure of the Nipah virus polymerase phosphoprotein complex. Nat Commun 15, 8673 (2024)" has been published, please re-evaluate the contribution of this manuscript.

Minor points:

1. The four copies of the P protein are difficult to distinguish due to their similar colors. To facilitate comparison with other nsNSVs, it is recommended to label them using other color strategy.
2. In Extended Data Figure 3a and Figure 2e, the yellow label "motif C" should be corrected to "motif D".
3. In Figure 3B and Extended Data Figure 6a, the term "RNA template" should be in blue.
4. Line 603: It should specify "20% polyacrylamide."

Other writing formats:

1. "Ptcls" should be changed to "particles" (Extended Data Figure 2b).
2. "GMPPnP" should be corrected to "GMPPNP" (Figure 3a and manuscript).
3. "MUMPS" should be revised to "MUV" (Extended Data Figure 7).
4. "Nipah" should be revised to "Nipah virus" (Line 633).
5. "TEV" should be clarified as "TEV protease" (Line 720).
6. "Mumps" should be corrected to "Mumps virus".
7. "uM" should be corrected to "μM" (Line 730).
8. Units (min/minutes, h/hours) and spacing should be corrected (e.g., Lines 228/720/762/767).

Reviewer #2

(Remarks to the Author)

The work by Sala et al. presents cryoEM structures of the Nipah virus polymerase-phosphoprotein complex in both apo and early replicating states. This work is largely a structural description with excellent resolution of the RNA and bound nucleotide analog in the replicating polymerase structure. This is an important work that strongly contributes to our understanding of nsNS RNA virus replication.

The enthusiasm for the structure determination of Nipah polymerase-P is somewhat lessened by a competing paper that published a nearly identical apo structure earlier this fall. However, the inclusion of RNA and an incoming nucleotide and, in particular, the clarity of these substrates in the density will make this an impactful work.

Major comments:

Mass spec methods are included, but mass spec results are only briefly mentioned and actual data is missing.

Minor comments:

ED Fig 1c, please increase the font size the +/- symbols to a legible size.

Fig 2b. As I understand it, this is the composite map of several reconstructions and this should be made clear in the figure legend. The consensus map was not included among the requested maps.

ED Fig 3b, labels for colored domains would help orient readers

Fig 5a, the color choices for the protein are poor here and it is hard to distinguish the two structures

Please give suppliers for the nucleotide analogs used in this study

Reviewer #3

(Remarks to the Author)

Nipah virus is a non-segmented negative-strand RNA virus (nsNSV) with a high pandemic potential in humans. Its RNA-dependent RNA polymerase (RdRp) complex is made by the L protein, a large multi-functional polypeptide that contains all required enzymatic activities, and its cofactor the phosphoprotein (P). The complex replicates and transcribes the viral genome and is so the ideal target to design new specific antivirals. Recent years have seen a profusion of nsNSV RdRp structures, some incomplete, some complete, with or without RNA mimicking the genomic RNA, most of them looking inactive. With this manuscript, Sala and co-workers present a structural analysis of Nipah RdRp, in apo and replicative conformations that constitutes a major achievement in the field. In particular, they can observe the position of an incoming nucleotides for the RNA synthesis in the catalytic core of the enzyme that has never been observed in any previous work. The manuscript is well written, describes perfectly the observations and therefore deserves to be published in Nature Communications.

Different aspects should also considered before acceptance.

Major

Regarding the manuscript, my major criticism concerns the way the manuscript is written, in particular how the authors consider the works of Grimes et al. (DOI: 10.21203/rs.3.rs-4663080/v1) and Yang et al. (Nat Commun; PMID: 39375338), published on the same subject 2024 July 12th and October 7th respectively. It means that the summary of the paper is not true, i.e. "However, to date no structural data is available on the NiV RdRp complex" as well as different sentences in the main text. If the three manuscripts (this one and the two already published) had a similar timeframe, this could have been perfectly acceptable, but that is not the case. The paper proposed by the authors bringing much more details than the two others do (I fully appreciate the quality of the work done), so the authors should take full advantage of this situation. As an example, Yang et al. with their incomplete structure speculate vaguely on the role of D832 for the transcription, whereas in the present active structure, this residue appears central for the RNA synthesis process and so crucial for the whole activity of the complex. Several aspects of the manuscript should therefore be reviewed to take these facts into account (with proper references).

The authors use the abbreviation "CAP" to refer solely to the GDP polyribonucleotidyltransferase domain (please add GDP line 59) that initiates the capping process at the 5' of the viral mRNA. Considering the biochemical aspect, especially the cap being the guanosyl moiety (added by the PRNTase activity) as well as the methyl in position 7 of the ring (added independently from the first activity by the methylase domain), I think the use of this abbreviation can be confusing. Furthermore, considering also that the RdRp of segmented NSV such as Orthomyxoviridae and/or Bunyaviridae having a Cap-binding domain, this could so be a second source of discrepancy with the use of such trivial/easy-to-use abbreviation.

Minor

- Please, check the names/abbreviations of the journals in the references: Example for ref 9 & 15 with PNAS with or without ".". Idem ref 5 & 30 with J Virol with or without ".". Please homogenize all the references.
- all Latin-sounding virus family names (Paramyxoviridae, Filoviridae, ...) might be in italics
- homogenize "%" and "°C". X% and X °C (with space between numbers and %) as well as X°C and X °C can be found!
- The Greek letter "gamma" used looks more a "y" than a real "γ". Also doubts for the "β"

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript has been significantly improved after revision. However, I do have two extra minors before the manuscript is appropriate for publication.

1. Line480-481, the authors provided the supplier for GMPPNP, but the suppliers of other nucleotides, especially fluorescein-labelled CTP, are still not listed. In addition, the concentration of fluorescently-labeled CTP was reported as 150 mM, while the concentrations of other nucleotides were stated to be 1 mM. Authors need to double check the concentrations of all these nucleotides utilized in the manuscript.

2. Line 443-444, the authors state the peak fractions that eluted from the heparin column were identified by SDS/PAGE (Extended Data Figure 1b). The coming question is whether the sample used for mass spectrometry was also from the heparin column elution rather than from the size exclusion chromatography (line 91). The SDS-PAGE shows two Phosphoprotein bands (band2 band3 in Extended Data Figure 1b) were present in heparin column elution. Does the elution fraction of the SEC still have these two bands?

Reviewer #2

(Remarks to the Author)

My concerns have been addressed.

Reviewer #3

(Remarks to the Author)

**Open Access** This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>



Response to reviewers

*Responses are in italique.*

Changes are marked in red in the manuscript file.

**Reviewer #1 (Remarks to the Author):**

In this study, the authors resolved two high-resolution structures of the NiV L-P complex in its apo and early-elongation states. The latter one represents a structural snapshot of an actively elongating L-P complex, with template RNA, product RNA, and incoming NTP bound, which is the highlight of the study. This finding helps enrich the understanding of nsNSV L-P complex during the RNA synthesis.

*We thank the reviewer for the positive evaluation and comments, which we addressed as outlined below.*

Major points:

1. Replication or transcription. The key finding of this work is to resolve the structure of NiV L-P complex with template RNA, product RNA, and incoming NTP bound. The authors claimed this state as the “replication” state throughout the manuscript including the title. Is there any evidence to strongly support this claim? The biochemical assay provided in the manuscript can only be claimed as “RNA synthesis”. The difference between genome replication and transcription mainly lies in the channel connectivity to the MTase and the RNA coated by nucleoprotein during replication. It will be great if authors compare the resolved structures with other nsNSV L-P complexes following the reference (Li, T. et al. Structures of the mumps virus polymerase complex via cryo-electron microscopy. Nat. Commun. 15, 4189 (2024)).

*We agree that our current biochemical data only demonstrate RNA synthesis and do not differentiate between replication or transcription. Since in both cases the L-P complex initiates RNA synthesis at the 3' leader sequence, the state we have resolved likely occurs during both processes before the polymerase has committed to one or the other. To reflect this, we now refer to this state as "actively elongating" throughout the manuscript and have changed the title of the paper accordingly.*

*As suggested by the reviewer, we have also included a comparison of the connectivity between the RdRp and MTase active sites in different nsNSV L-P complexes (new Extended Data Figure*

8) and have extended the corresponding parts of the results section. In addition, we have also added a paragraph to the discussion in which we emphasize the early nature of the elongation complex resolved in this study.

2. RNA in the apo-state NiV L-P complex. In the extended data Figure 5, authors also found the apo-state NiV L-P complex (the ratio at 32%) in the whole dataset. It is not clear whether there is RNA, no matter template RNA or product RNA, resolved in this structure. If not, RNA may play an important role in determining more density. Can authors increase the ratio of NiV L-P with the appendage (CTD map) via elevating RNA ratio?

*Indeed, we found that a substantial proportion of the particles in this dataset represent the apo state. The reconstruction from these is identical to the apo reconstruction from the first dataset, with no indications of either RNA template or product. To emphasize this, we now clearly indicated this in bold in Extended Figure 5.*

*Regarding the question of the RNA and extra density, we indeed observe ordered density for the C-terminal domain in all particles with RNA present. We used a 15-fold molar excess of RNA relative to the L-P complex during complex preparation for cryo-EM, but it is very well possible that the proportion of NiV L-P particles with ordered CTDs could be increased by incubating with an even larger excess of RNA. However, we did not explore this possibility experimentally as the number of particles we obtained with RNA bound was sufficient to generate a high-resolution reconstruction.*

3. Nomenclature. It is confused to use NiV L-P complex as NiV RdRp complex, since L has a RdRp domain. The same nomenclature issue occurs on P. Four P molecules in P tetramer are usually depicted as P1, P2, P3, and P4, respectively. It is better to keep consistent with other papers in the field.

*We agree with the reviewer that it is best to keep a consistent nomenclature across the literature. We have thus adopted the naming convention of labeling the four P molecules as P1, P2, P3, and P4 and refer to the complex as NiV L-P complex, in line with other publications.*

4. Line 22/350: The article of “Yang, G., Wang, D. & Liu, B. Structure of the Nipah virus polymerase phosphoprotein complex. Nat Commun 15, 8673 (2024)” has been published, please re-evaluate the contribution of this manuscript.

*We agree with the reviewer that this work needs to be accounted for. We would like to clarify that this paper was published after we wrote and first submitted our manuscript, and it was*

*thus not cited and discussed. We have revised our manuscript to reflect the current state of the literature, also including two further recently published papers on the apo NiV L-P complex (PMID 39661676, 39627254).*

Minor points:

1. The four copies of the P protein are difficult to distinguish due to their similar colors. To facilitate comparison with other nsNSVs, it is recommended to label them using other color strategy.

*We thank the reviewer for pointing this out. We have changed the coloring such that there is more contrast between the colors to more clearly distinguish between the P monomers.*

2. In Extended Data Figure 3a and Figure 2e, the yellow label "motif C" should be corrected to "motif D".

*We thank the reviewer for pointing this out and have corrected it.*

3. In Figure 3B and Extended Data Figure 6a, the term "RNA template " should be in blue.

*We thank the reviewer for pointing this out. We have correct the figure.*

4. Line 603: It should specify "20% polyacrylamide."

*We thank the reviewer for pointing this out and have corrected it.*

Other writing formats:

1. "Ptcls" should be changed to "particles" (Extended Data Figure 2b).

*We have changed this as suggested.*

2. "GMPPnP" should be corrected to "GMPPNP" (Figure 3a and manuscript).

*We have changed this as suggested.*

3. "MUMPS" should be revised to "MUV" (Extended Data Figure 7).

*We have changed the Figure as suggested.*

4. "Nipah" should be revised to "Nipah virus" (Line 633).

*We have revised this as suggested.*

5. "TEV" should be clarified as "TEV protease" (Line 720).

*We have changed this as suggested.*

6. "Mumps" should be corrected to "Mumps virus".

*We have changed this as suggested.*

7. "uM" should be corrected to " $\mu$ M" (Line 730).

*We have corrected this.*

8. Units (min/minutes, h/hours) and spacing should be corrected (e.g., Lines 228/720/762/767).

*We have corected this.*

Reviewer #2 (Remarks to the Author):

The work by Sala et al. presents cryoEM structures of the Nipah virus polymerase-phosphoprotein complex in both apo and early replicating states. This work is largely a structural description with excellent resolution of the RNA and bound nucleotide analog in the replicating polymerase structure. This is an important work that strongly contributes to our understanding of nsNS RNA virus replication.

The enthusiasm for the structure determination of Nipah polymerase-P is somewhat lessened by a competing paper that published a nearly identical apo structure earlier this fall. However, the inclusion of RNA and an incoming nucleotide and, in particular, the clarity of these substrates in the density will make this an impactful work.

*We thank the reviewer for their positive evaluation!*

Major comments:

Mass spec methods are included, but mass spec results are only briefly mentioned and actual data is missing.

*In the revised manuscript, we now include mass spec results in Extended Data Figure 1.*



Minor comments:

ED Fig 1c, please increase the font size the +/- symbols to a legible size.

*We have increased the font size as suggested.*

Fig 2b. As I understand it, this is the composite map of several reconstructions and this should be made clear in the figure legend. The consensus map was not included among the requested maps.

*We have revised the figure legend to clearly state that a composite map is shown. We apologize that the consensus map was not among the files provided, and have now uploaded it together with the remaining files if the reviewer wishes to inspect it.*

ED Fig 3b, labels for colored domains would help orient readers

*We have now included labels in the figure.*

Fig 5a, the color choices for the protein are poor here and it is hard to distinguish the two structures

*We have revised the colors in this figure to higher contrast to improve the interpretability.*

Please give suppliers for the nucleotide analogs used in this study

*We now include the supplier for the GMPPNP used.*

Reviewer #3 (Remarks to the Author):

Nipah virus is a non-segmented negative-strand RNA virus (nsNSV) with a high pandemic potential in humans. Its RNA-dependent RNA polymerase (RdRp) complex is made by the L protein, a large multi-functional polypeptide that contains all required enzymatic activities, and its cofactor the phosphoprotein (P). The complex replicates and transcribes the viral genome and is so the ideal target to design new specific antivirals. Recent years have seen a profusion of nsNSV RdRp structures, some incomplete, some complete, with or without RNA mimicking the genomic RNA, most of them looking inactive. With this manuscript, Sala and co-workers present a structural analysis of Nipah RdRp, in apo and replicative conformations that constitutes a major achievement in the field. In particular, they can observe the position of an incoming nucleotides for the RNA synthesis in the catalytic core of the enzyme that has never been observed in any previous work. The manuscript is well written, describes perfectly the observations and therefore deserves to be published in Nature Communications.

*We thank the reviewer for the positive evaluation!*

Different aspects should also considered before acception.

Major

Regarding the manuscript, my major criticism concerns the way the manuscript is written, in particular how the authors consider the works of Grimes et al. (DOI: 10.21203/rs.3.rs-4663080/v1) and Yang et al. (Nat Commun; PMID: 39375338), published on the same subject 2024 July 12th and October 7th respectively. It means that the summary of the paper is not true, i.e. “However, to date no structural data is available on the NiV RdRp complex” as well as different sentences in the main text. If the three manuscripts (this one and the two already published) had a similar timeframe, this could have been perfectly acceptable, but that is not the case.

*We agree with the reviewer that the manuscript should appropriately reflect the state of the literature. During the preparation of our manuscript, two preprints were posted, both describing the structure of the apo NiV RdRp complex: Grimes et al. (2024) and Abraham and Fearn et al (2024) (<https://doi.org/10.1101/2024.05.29.596445>). Since none of them had been published as peer-reviewed articles by the time we submitted our work, we cited them and considered our work to be complementary and in approximately the same timeframe. The*

*manuscript by Yang et al. (PMID: 39375338) was published in the same week we submitted our manuscript to Nature Communications and after we posted our work as preprint on bioRxiv, and we were hence not aware of this paper during the preparation of our manuscript. In the revised manuscript, we now cite all three other papers as well as two additional papers on the apo NiV L-P complex that were published in December 2024 (PMID 39661676, 39627254). We have also revised our wording in the summary and main text to emphasize that the novelty of our works lies in the RNA-bound structure.*

The paper proposed by the authors bringing much more details than the two others do (I fully appreciate the quality of the work done), so the authors should take full advantage of this situation. As an example, Yang et al. with their incomplete structure speculate vaguely on the role of D832 for the transcription, whereas in the present active structure, this residue appears central for the RNA synthesis process and so crucial for the whole activity of the complex. Several aspects of the manuscript should therefore be reviewed to take these facts into account (with proper refereneecs).

*We thank the reviewer for this suggestion and have revised our manuscript to take into account the existing literature. In particular, we now discuss the results by Yang et al and others regarding D832 in the light of our structure.*

The authors use the abbreviation “CAP” to refer solely to the GDP polyribonucleotidyltransferase domain (please add GDP line 59) that initiates the capping process at the 5’ of the viral mRNA. Considering the biochemical aspect, especially the cap being the guanosyl moiety (added by the PRNTase activity) as well as the methyl in position 7 of the ring (added independently from the first activity by the methylase domain), I think the use of this abbreviation can be confusing. Furthermore, considering also that the RdRp of segmented NSV such as Orthomyxoviridae and/or Bunyaviridae having a Cap-binding domain, this could so be a second source of discrepancy with the use of such trivial/easy-to-use abbreviation.

*We agree with the reviewer that the designation “CAP” does not fully reflect the biochemical function of this domain, as it only carries out one step of the capping reaction. We have thus changed this and now refer to this domain as “PRNTase”.*

Minor

- Please, check the names/abbreviations of the journals in the references: Example for ref 9 & 15 with PNAS with or without “.”. Idem ref 5 & 30 with J Virol with or without “.”. Please homogenize all the references.

*We have corrected and homogenized the references.*

- all Latin-sounding virus family names (Paramixoviridae, Filoviridae, ...) might be in italics

*We have adopted this.*

- homogenize “%” and “°C”. X% and X % (with space between numbers and %) as well as X°C and X °C can be found!

*We have harmonized this.*

- The Greek letter “gamma” used looks more a “y” than a real “γ”. Also doubts for the “β”

*We have corrected this.*

Response to the reviewers

*Responses are in italique.*

Changes are marked in red in the manuscript file.

**Reviewer #1 (Remarks to the Author):**

The manuscript has been significantly improved after revision. However, I do have two extra minors before the manuscript is appropriate for publication.

*We thank the reviewer for the carefull evaluation.*

1. Line480-481, the authors provided the supplier for GMPPNP, but the suppliers of other nucleotides, especially fluorescein-labelled CTP, are still not listed. In addition, the concentration of fluorescently-labeled CTP was reported as 150 mM, while the concentrations of other nucleotides were stated to be 1 mM. Authors need to double check the concentrations of all these nucleotides utilized in the manuscript.

*We have now included the supplier and concentrations for all the nucleotides used.*

2. Line 443-444, the authors state the peak fractions that eluted from the heparin column were identified by SDS/PAGE (Extended Data Figure 1b). The coming question is whether the sample used for mass spectrometry was also from the heparin column elution rather than from the size exclusion chromatography (line 91). The SDS-PAGE shows two Phosphoprotein bands (band2 band3 in Extended Data Figure 1b) were present in heparin column elution. Does the elution fraction of the SEC still have these two bands?

*In fact, the mass spectrometry and SDS-PAGE shown in Extended Data Figure 1b correspond to the protein concentrated after SEC, which still contains the two bands. The heparin-purified sample showed degradation, but the SDS-PAGE for this step was not displayed. We have revised the text to improve clarity.*

**Reviewer #2 (Remarks to the Author):**

My concerns have been addressed.