

Alphavirus nsP3 organizes into tubular scaffolds essential for infection and the cytoplasmic granule architecture

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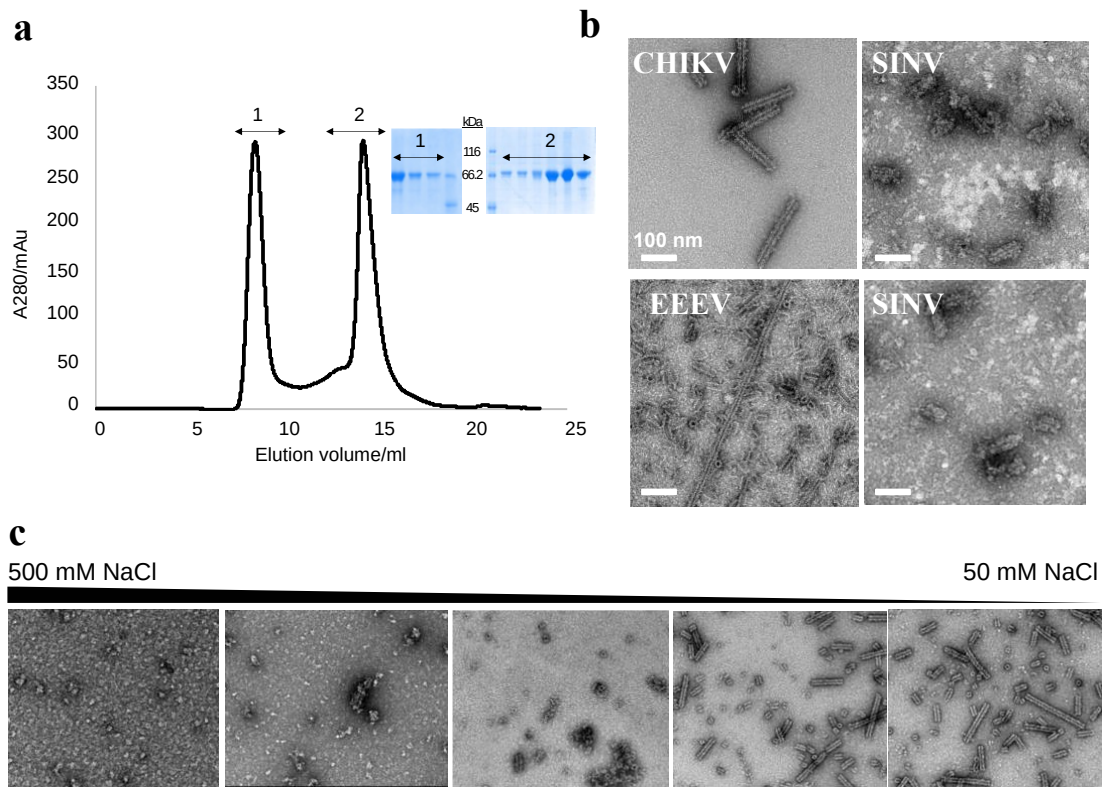
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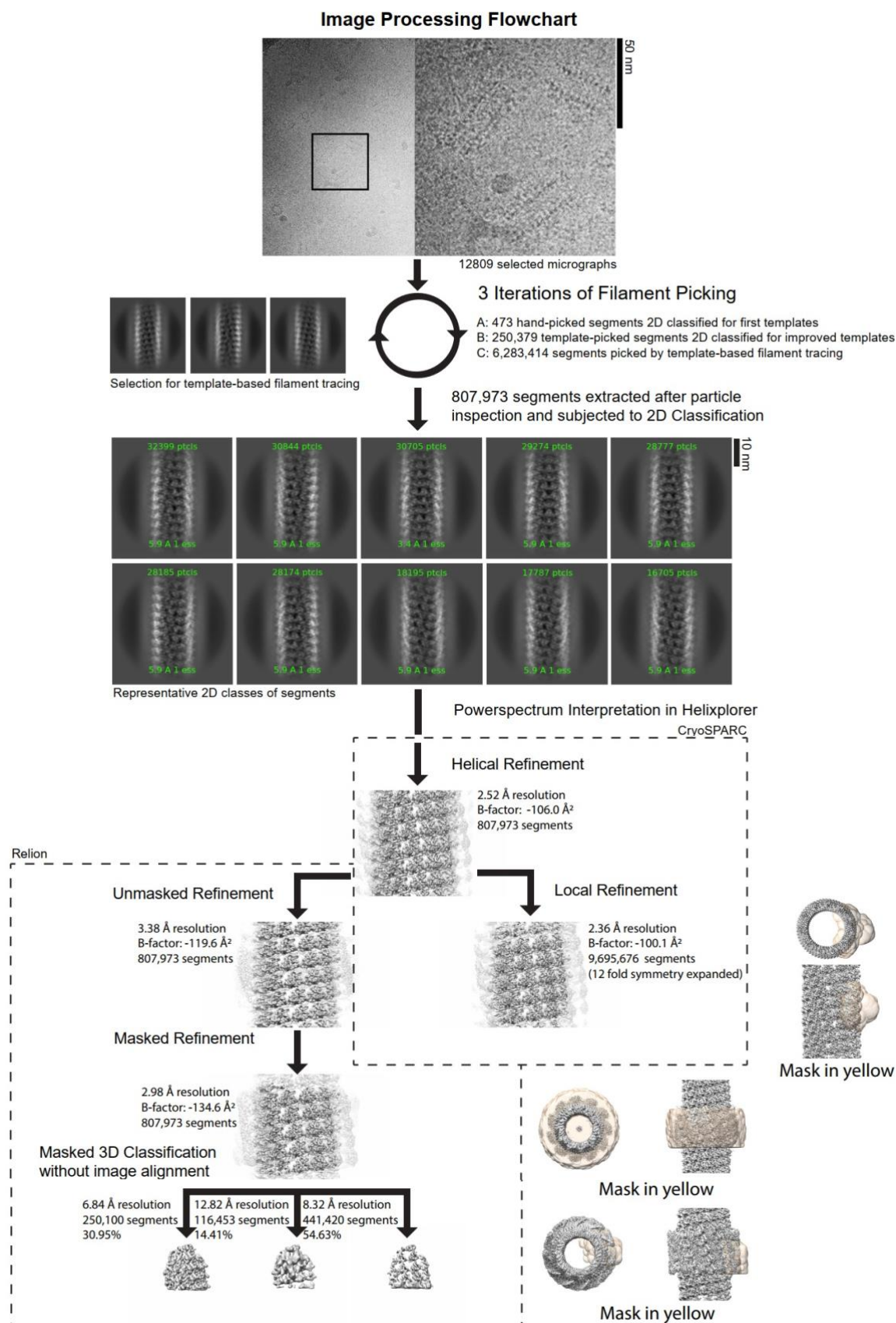
This PDF file includes:

Supplementary Figs. 1 to 10

Source Data for Supplementary Figs. 7, 9 and 10

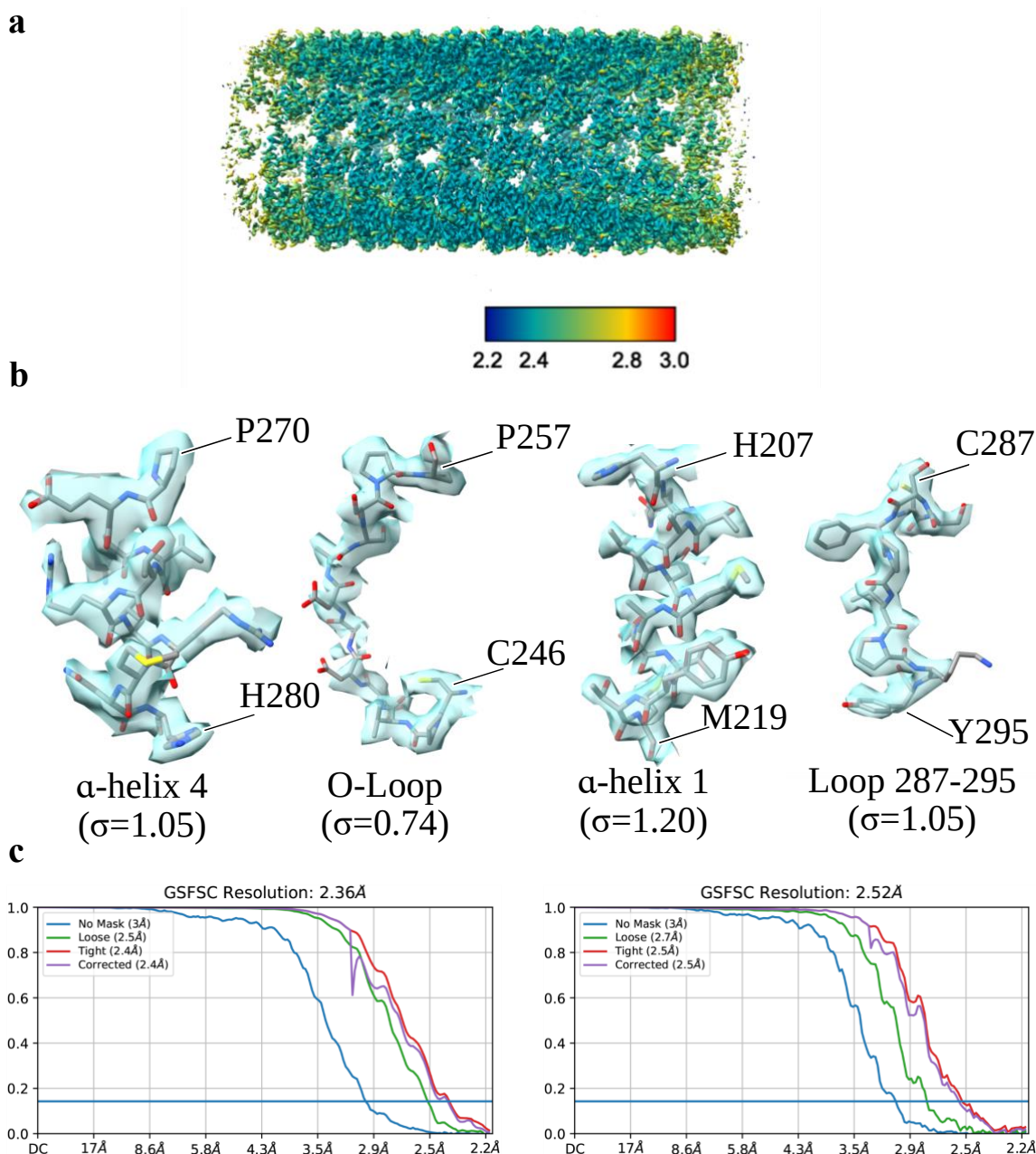


Supplementary Fig. 1. CHIKV nsP3 purification and electron microscopy analysis. **a**, Size exclusion chromatography profile of nsP3 protein expressed in insect cells after nickel affinity chromatography at 0.5 M NaCl. Two picks are obtained, one at high molecular weight (1) and another consistent with monomeric nsP3 (2). The inset shows an SDS-PAGE of the indicated fractions for both peaks 1 and 2 showing the CHIKV nsP3 migrated at the expected molecular weight in both cases (61kDa). **b**, Negative staining TEM micrograph of the sample from the peak 1 of panel a, showing the tubular structures formed by CHIKV, SINV or EEEV purified nsP3 proteins. **c**, The monomeric CHIKV nsP3 was diluted to 0.2 mg/ml in buffers with decreasing NaCl concentration and equilibrated for 1h at 4°C. The CHIKVnsP3 assemblies start to appear at 200 M NaCl concentration.

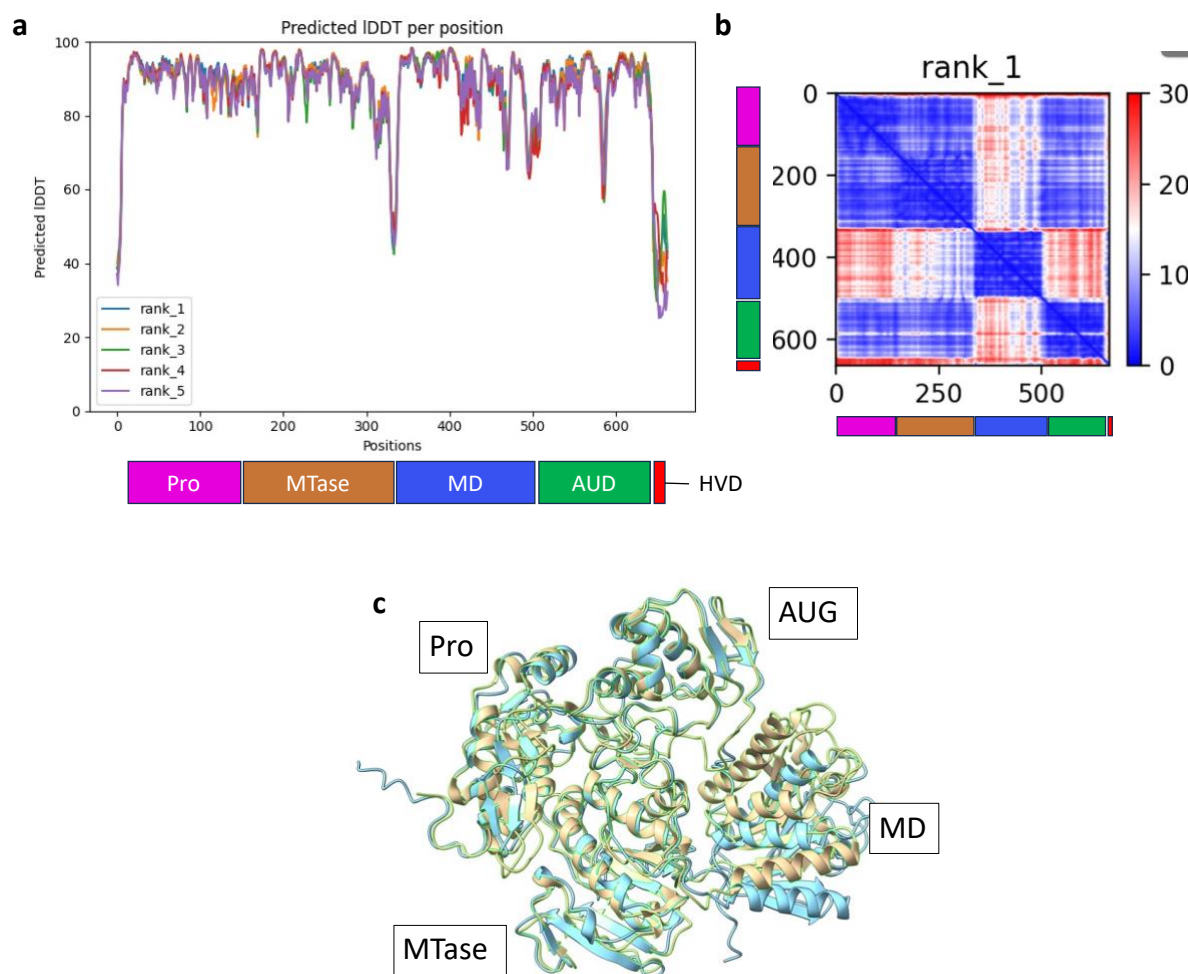


Supplementary Fig. 2. Flowchart of the image processing pipeline for nsP3. A representative micrograph section is shown with two different magnifications with exemplary nsP3 particle segments. The iterative segment picking workflow is

illustrated. Particles are extracted and representative class averages are shown. Helical parameters were defined in helixplorer software (<https://rico.ibs.fr/helixplorer>, see supplementary information). Unmasked and masked refinement were performed in cryoSPARC and Relion with subsequent local classification procedures. 3D maps are represented in greyscale with two different thresholds to show both the densities of AUDs and macrodomains. Final maps are shown with annotated overall resolutions and B-factors. Used software packages are indicated. For a detailed description of the image processing pipeline, see methods, Table 1 and supplementary information.

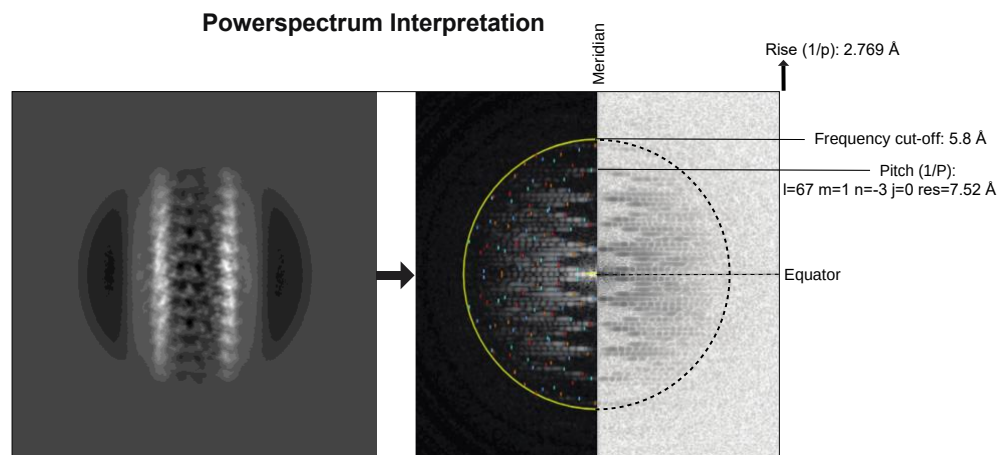


Supplementary Fig. 3. Quality and resolution of final Cryo-EM maps. **a**, Local resolution map for the EMD: 17678, the color key shows the color code at the indicated resolutions in Å. **b**, Representative fragments of electron density maps (EMD: 17678) are shown in transparent cyan at the specified sigma. The refined model (PDB: 8PHZ) is represented in sticks and colored by atom. The first and last residues for each fragment are labeled. **c**, Fourier shell correlation curves for the helical refinement and the focused refinement maps in the left and right panels respectively (EMD: 17729 and EMD: 17678, respectively).

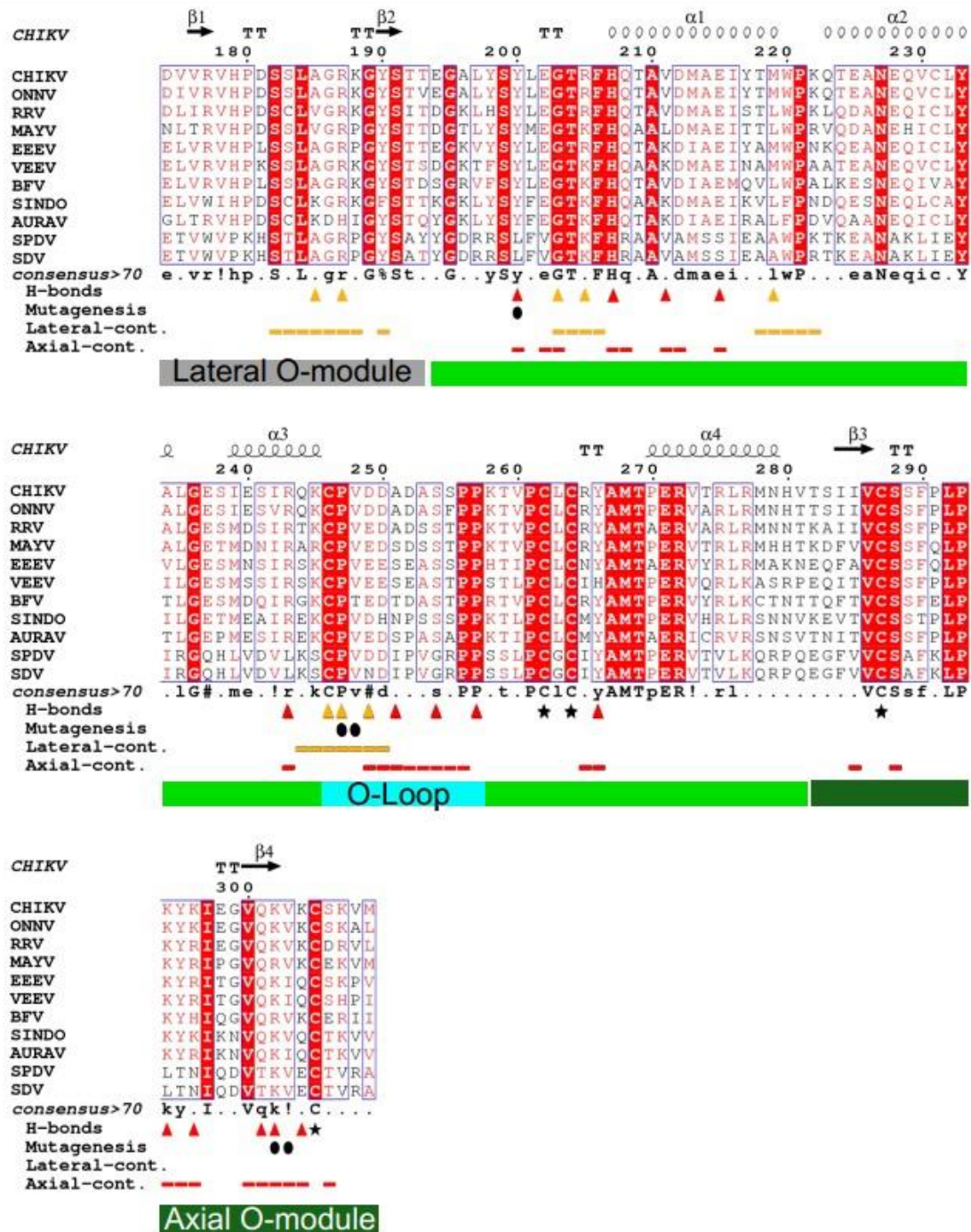


Supplementary Fig. 4: AlphaFold2 structure prediction of CHIKV nsP2-3.

Statistics for the alphafold2 prediction for CHIKV nsP2-3 uncleaved form including the nsP2 protease (Pro), Methyl transferase (MTase) and the nsP3 macrodomain (MD), the alphavirus unique domain (AUD) and the start of the hypervariable domain (HVD). **a**, Per-residue confidence coloring (pLDDT) plot of 5 predicted models covering the sequence numbered from the beginning of the protease domain. The domains are indicated and colored by bars at the bottom of the plot following the same coloring as in Fig. 1. Most of the model is confidently predicted with pLDDT values above 70. **b**, Residue-residue alignment confidence (PAE) plot of the predicted model ranked 1 with the best pLDDT. The domains are also indicated in the X and Y axis as in panel **a**. The prediction shows confident conformation between the Pro, MTase and AUD domains with rmsd values below 10. The plot also shows that the relative position of the macrodomain with the other three domains is not confidently predicted. The predicted structure is very similar to the equivalent experimental structure reported for SINV (see main text). **c**, Superposition of SINV nsP2-3 structure (PDB: 4AUG) and the AlphaFold2 predicted structure for CHIKV. The structures are represented as cartoons and colored wheat and cyan respectively. The domains are labeled as in panel **a**.

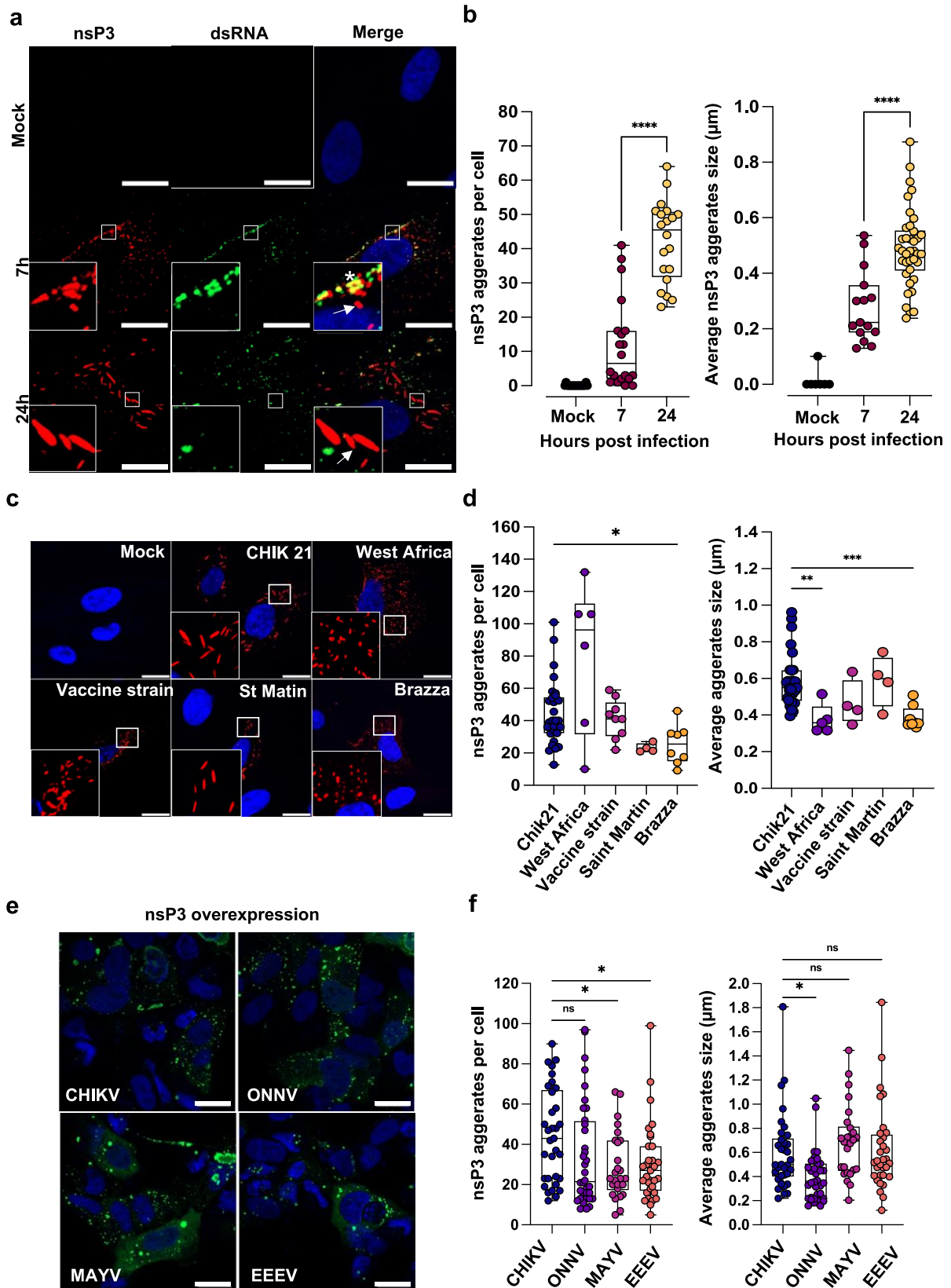


Supplementary Fig. 5. Powerspectrum Interpretation. Illustration of a representative class averages used for powerspectrum interpretation in helixplorer software (<https://rico.ibs.fr/helixplorer>). Potential solutions of helical parameters (helical rise and twist) were tested by 3D helical refinement in cryoSPARC. Helical parameters were refined during 3D reconstruction and converged to a twist of -164.175 degrees and a helical rise of 2.782 Å. The layerline defined as the pitch (1/P: $l=67$ $m=1$ $n=-3$ $j=0$ $res=7.52$ Å) is highlighted within the powerspectrum. The best defined symmetric and representative class average was selected by visual inspection, rescaled and used for the calculation of a powerspectrum (bshow) in helixplorer software varying combinations of helical pitch and helical rise values, giving a list of potential symmetry parameters validated by visual inspection. These were tested as initial symmetry parameters for cryoSPARC helical refinement. In order to narrow down the search range for helixplorer, we made the following hypotheses. Initially, the layer line at 7.52 Å with a maximum near the meridian was attributed the rise but although several good matches between predicted and experimental power spectra were found in helixplorer the cryoSPARC helical refinement ruled out this hypothesis. We then hypothesized that this layer line corresponded to the pitch and one (1: Rise 2.769, Twist -164.175) of four tested helical models (with 2: Rise 2.528, Twist 150.380; 3: Rise 2.353, Twist 138.736; 4: Rise 2.162, Twist 128.207) refined to high resolution.



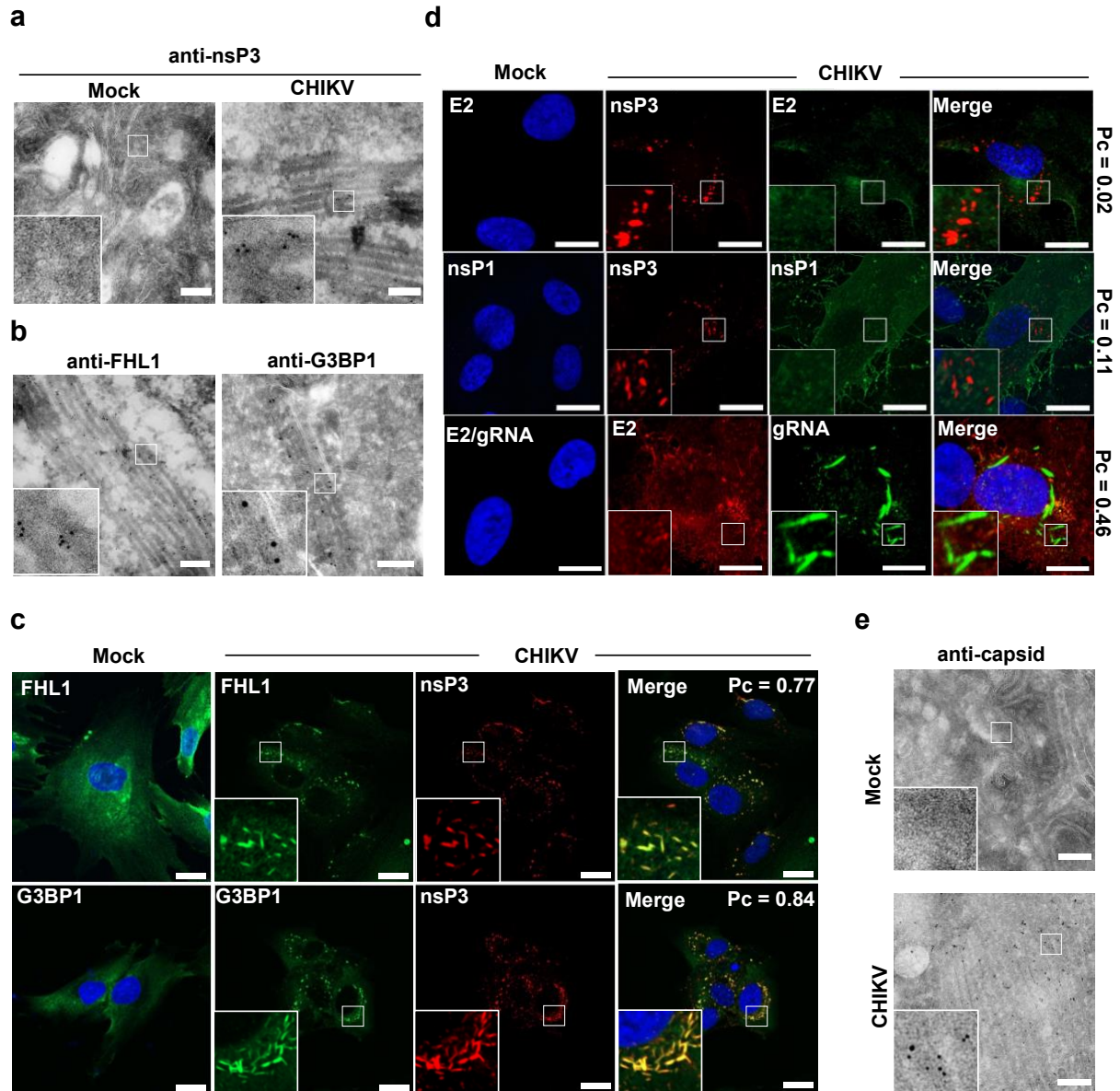
Supplementary Fig. 6. Sequence alignment of the AUD from different alphaviruses. ESPript3 sequence alignment representation of CHIKV (UniProt: Q8JUX6), O'nyong-nyong virus (ONNV) (UniProt: P13886), Ross river virus (RRV) (UniProt: P13887), Mayaro virus (MAYV) (UniProt: Q8QZ73), Eastern equine encephalitis virus (EEEV) (UniProt: Q4QXJ8), Venezuelan equine encephalitis virus (VEEV) (UniProt: P36327), Barmah forest virus (BFV) (UniProt: P87515), Sindbis virus (SINDO) (UniProt: P03317), Aura virus (AURAV) (UniProt: Q86924) and Sleeping disease virus (SDV) (UniProt: Q8JJX1). The secondary structures of the AUD are indicated in the top and residue numbering corresponds to CHIKV. At the bottom the

consensus sequence is displayed, the residues maintaining hydrogen bonds in the lateral and axial interfaces are indicated by yellow and red triangles, respectively, and cysteine residues coordinated with the Zn atom are labelled by stars. Those residues maintaining contacts in the lateral and axial interfaces are indicated by yellow and red bars, respectively. The contacts were calculated by the PISA server and alignment done with TCOFFEE (EBI). The mutated residues in this study with drastic effects on replication and alpha-granule formation are indicated by black dots. The regions forming the O-modules and O-loop are indicated by thick bars at the bottom of the alignment following the same colour code as in Fig. 1d.



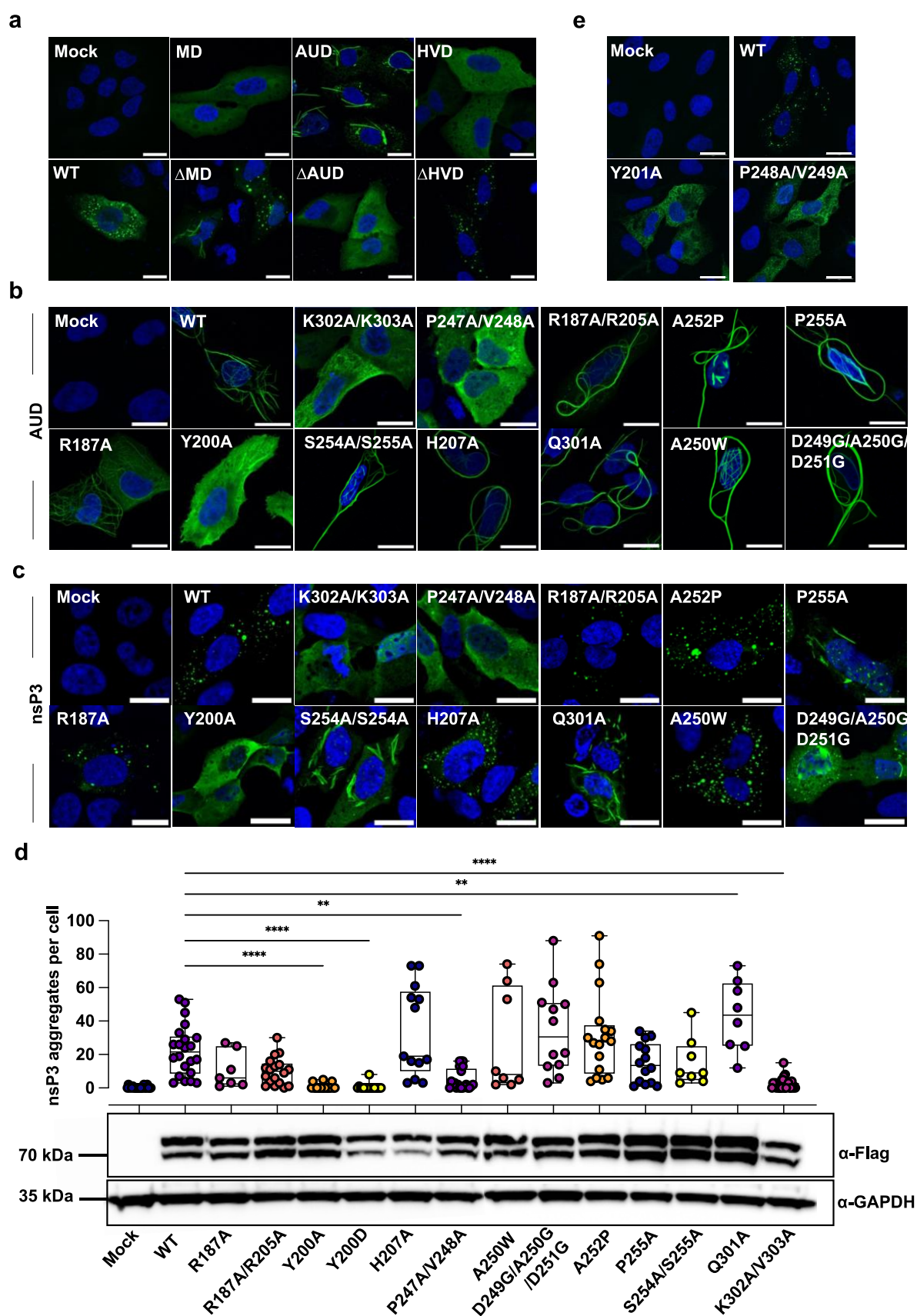
Supplementary Fig.7. NsP3 alpha-granules and alphavirus infection. a,b, Human fibroblasts were inoculated with the CHIK21 strain for 7 h (MOI of 10) or 24 h (MOI 2).

Mock and infected cells were co-stained with a mouse anti-dsRNA mAb and rabbit anti-nsP3 Ab. Representative images from 2 independent experiments were acquired by confocal microscopy and are shown. Replicative complexes (asterisk) and nsP3 alpha-granules (arrow) are shown. Co-localization analysis were performed as described in Fig 3b. Images are representative from 2 independent experiments. Scale bars, 20 μ m. **b.** nsP3-aggregates were quantified following image segmentation by signal thresholding. Graph bar showing the number of nsP3-aggregates per cell (left) and the average aggregate size (right) from two independent biological replicates (n=20 cells in mock, n=20 cells at 7 h.p.i and n=20 cells at 24 h.p.i). h.p.i, hours post-infection. Data are presented as min to max with all data points shown and adjusted *P*-values are calculated by one-way ANOVA with a Dunnett's multiple comparison test (**** $P < 0.0001$). **c, d,** Human fibroblasts were inoculated either with CHIKV21, West Africa, vaccine strain 181/25, Saint Martin or Brazza CHIKV strains for 24 h. **c,** nsP3 alpha-granule was detected using anti- nsP3 specific Ab and images were acquired by confocal microscopy. Data are representative from 2 independent experiments. **d,** The number of alpha-granule per cell (left plot) and the average aggregate size (right plot) were quantified (n=30 cells in CHIKV21, n=6 cells for West Africa, n=9 cells for Vaccine strain, n=4 cells for St Martin and n=8 cells for Brazza strain). Data are presented as min to max with all data points shown and adjusted *P*-values are calculated by one-way ANOVA with Kruskal-Wallis multiple comparisons test (** $P = 0.0008$, ** $P = 0.0053$, * $P = 0.0350$). **e,** U2OS cells were transfected for 48 hours with FLAG-tagged CHIKV-nsP3, ONNV-nsP3, MAYV-nsP3 or EEEV-nsP3 expressing plasmids. Alpha-granules were detected by confocal microscopy following anti-FLAG immunostaining. Images are representative from 2 independent experiments. Scale bars, 20 μ m. **f,** The number of aggregates per cell (left plot) and the average aggregate size (right plot) were quantified as in **b**, (n=24 cells in CHIKV, n=18 cells in ONNV, n=12 cells in MAYV and n=12 cells in EEEV). Data are presented as min to max with all data points shown and adjusted *P*-values are calculated by one-way ANOVA with a Dunnett's multiple comparison test (* $P = 0.0251$). Source data are provided as a Source Data file.



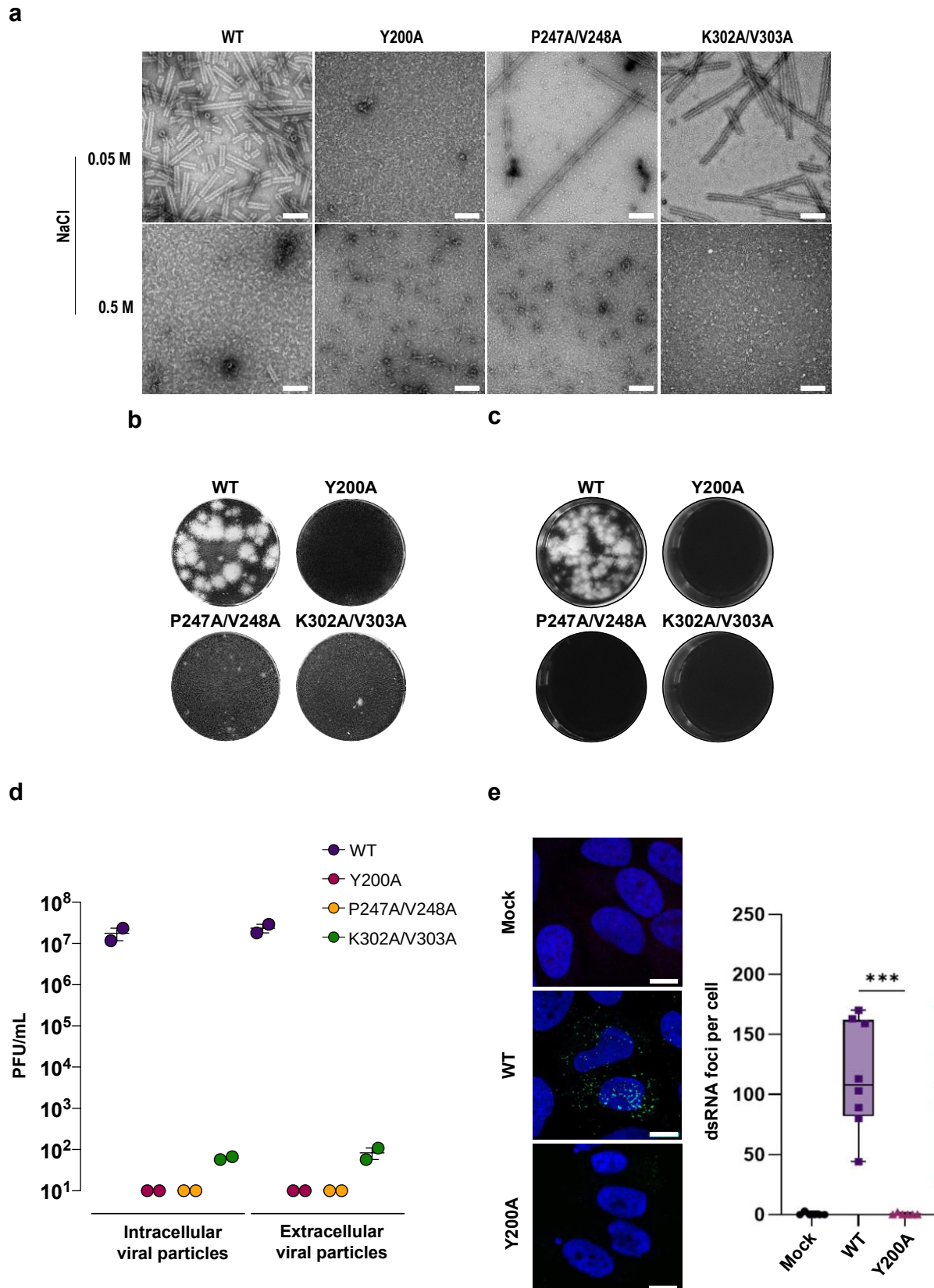
Supplementary Fig.8. NSP3 tubular structure characterization. **a**, Transmission electron microscopy of CHIKV-infected fibroblasts following immunogold labeling with an anti-nsP3 antibody. Scale bars, 200 nm. **b**, Transmission electron microscopy of CHIKV infected human dermal fibroblasts following immunostaining using gold anti-FHL1 or -G3BP1 Ab, showing that nsP3 tubular structures contain FHL1 and G3BP1. Scale bars, 200 nm. **c**, Human fibroblasts were inoculated with CHIK-nsP3-mCherry virus at MOI of 5 for 20 hours and immunostained for FHL1 and G3BP1 expression. Images were acquired by confocal microscopy (nsP3-mCherry in red, FHL1 and G3BP1 in green). Co-localization analysis were performed as described in Fig 3b. Pearson's coefficients (Pc) are shown for each staining (n>15 cells). Scale bars, 20 μ m. **d**, Upper and middle panel : human dermal fibroblasts were infected with CHIKV cherry as in **c**, and stained for E2 and nsP1 expression using specific Ab (green). Co-

localization analysis were performed as described in **c**. Lower panel : human dermal fibroblasts were infected with CHIKV 21 and stained for E2 (red). CHIKV genomic RNA was detected following incubation with CHIKV specific fluorescent RNA probes (green channel). Co-localization analysis were performed as described in **c**. **e**, Transmission electron microscopy of CHIKV-infected fibroblasts following immunogold labeling with an anti-capsid antibody. Scale bars, 200 nm. (**a-e**) Images are representative of 3 independent experiments.



Supplementary Fig. 9. The AUD mediates nsP3 oligomerization. a, U2OS cells were transfected with plasmids encoding FLAG-tagged CHIKV nsP3, nsP3

Macrodomain (MD), nsP3 Alphavirus unique domain (AUD), nsP3 Hypervariable domain (upper panels), or CHIKV nsP3 deleted for one of these domains (Δ MD, Δ AUD or Δ HVD) (lower panels). At 48h after transfection, cells were fixed and stained with FLAG antibody. Images were acquired by confocal microscopy and are representative of 2 independent experiments. Scale bars, 20 μ m. Images are representative of 2 independent experiments. **b**, U2OS cells were transfected with either FLAG-tagged wild-type nsP3 AUD or a panel of nsP3 AUD constructs harboring mutations of residues proposed to mediate interprotomer contacts (see Fig. 1). AUD filaments formation was assessed 48 h after transfection by immunostaining with FLAG antibody. Images were acquired by confocal microscopy and are representative of 2 independent experiments. Scale bars, 20 μ m. **c-d**, U2OS cells were transfected with the full-length FLAG-tagged CHIKV nsP3 carrying the AUD mutations shown in **b**. **c**, The ability of each mutant to form cytoplasmic aggregates was assessed by immunostaining with FLAG antibody. Images are representative of 2 independent experiments. Scale bars, 20 μ m. **d**, Graph bar showing the number of nsP3-aggregates per cell from 2 independent experiments ($n \geq 15$ cells). Data are presented as min to max with all data points shown and adjusted P -values are calculated by one-way ANOVA with a Dunnett's multiple comparison test (**** $P < 0.0001$, ** $P = 0.0021$). In the bottom panel is shown NsP3 expression in U2OS transfected cells. **e**, BHK cells were transfected with Flag-tagged WT, Y201A or P248A/V249A SINV nsP3 plasmids. Intracellular nsP3 localization was assessed by confocal microscopy using an anti-Flag mAb. Data are representative of two independent experiments. Source data are provided as a Source Data file.



Supplementary Fig. 10. Role of nsP3 helical scaffolds during CHIKV infection. a, Negative electron TEM images of nsP3 filaments formed by WT and mutant nsP3 proteins at 0.05 M and 0.5 M NaCl after 1h incubation at RT. **b-c,** 293T cells (b) or

U2OS cells (c) were transfected with the indicated capped *in vitro* transcribed CHIKV RNA. Supernatant of infected cells were collected at 48 h post transfection and viral titers were measured by plaque assay on VeroE6 cells. Images representative of WT (supernatant dilution at 1/1000) and mutant virus plaques (at supernatant dilution of 1/2) are shown. Data are representative of independent experiments performed in duplicate. **d**, U2OS were transfected as described in **c**. At 48h post transfection, extracellular and intracellular infectious particles were quantified by plaque assay on VeroE6 cells. Data from one experiment representative of 2 independent experiments done in duplicate are presented as mean value $\pm$ SD. **e**, U2OS cells were transfected with the indicated CHIKV RNA. 48H later, cells were permeabilized and stained for replication complex formation using an anti-dsRNA Ab. dsRNA foci were quantified following image segmentation and signal thresholding using ImageJ. Graph bar showing the number of dsRNA-foci per cell. Data are presented as min to max with all data points shown (n=6 cells in mock, n=8 cells in WT, n=6 in Y200A) and adjusted *P*-values are calculated by one-way ANOVA with a Dunnett's multiple comparison test (***) $P < 0.0002$). Source data are provided as a Source Data file.