

Two-dimensional HRS condensates drive the assembly of flat clathrin lattices on endosomes

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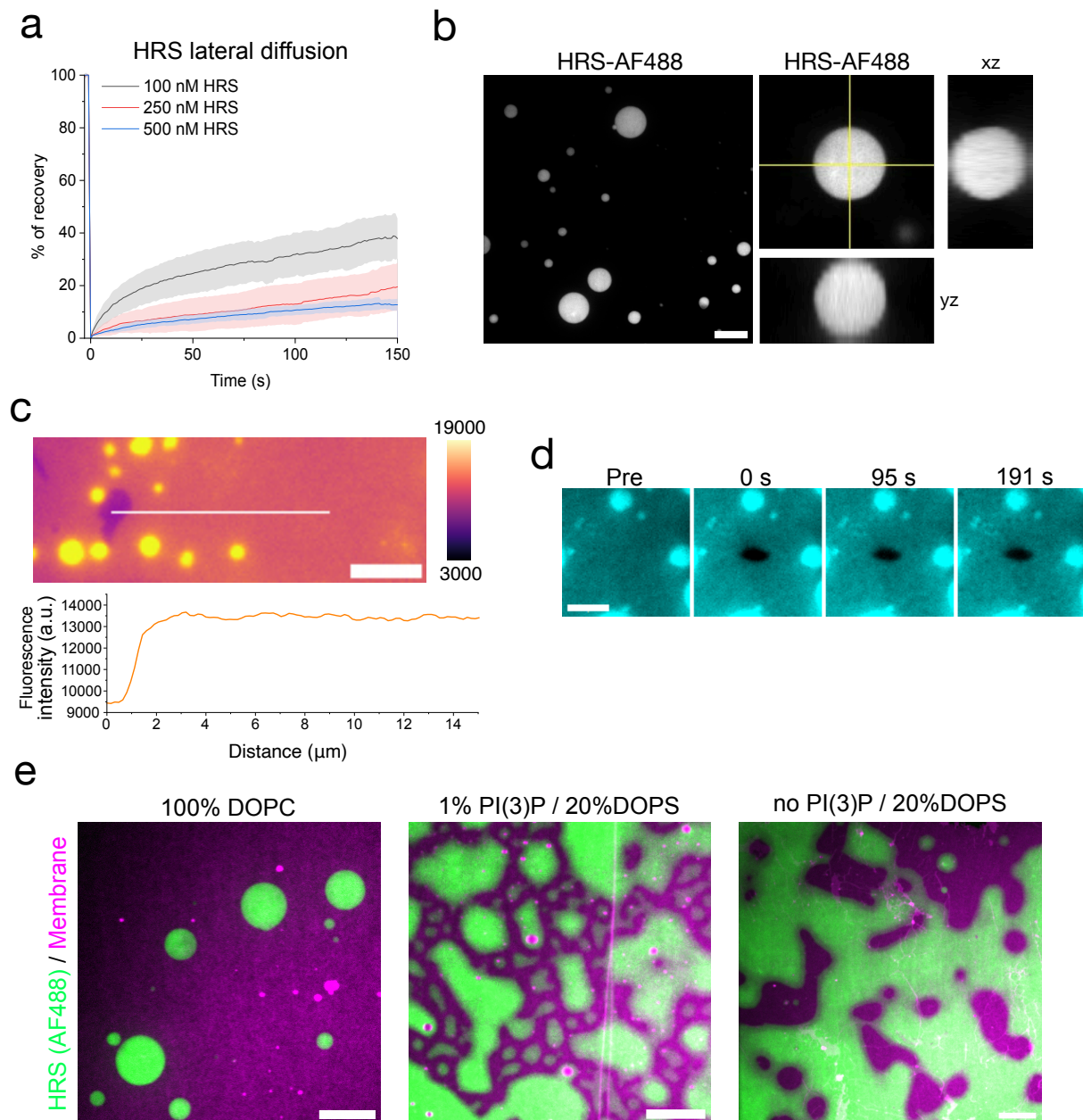
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Supplementary information

- Supplementary figures 1-10
- Supplementary Tables 1-4
- Uncropped Western blots

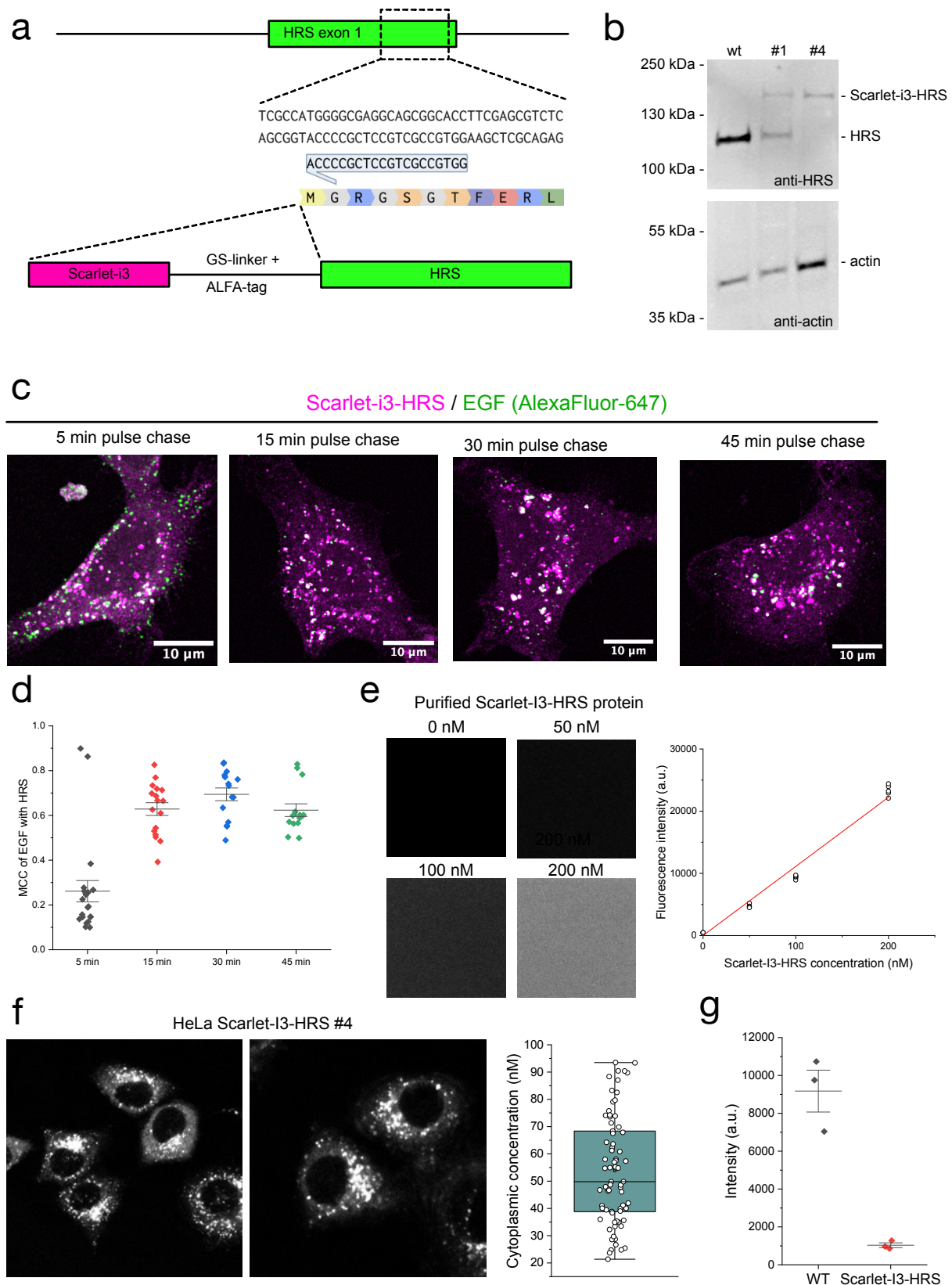
Supplementary Figures



Supplementary Figure 1

a, FRAP of AlexaFluor-568 labeled HRS on the SLB. Data is a mean of 20 measurements (100 nM HRS), 18 measurements (250 nM HRS), and eight measurements (500 nM HRS) with standard deviations shown. **b**, Representative fluorescence microscopy images of 2 μM AlexaFluor-488 labeled HRS in 20 mM HEPES, pH 7.2, 125 mM potassium acetate, and 1 mM magnesium acetate. The scale bar is 10 μm . Orthogonal views of HRS condensate are shown. **c**, A fluorescent microscopy image of HRS condensate wetting the membrane with a fluorescence intensity heatmap and a line profile analysis of HRS fluorescence intensity over the condensate.

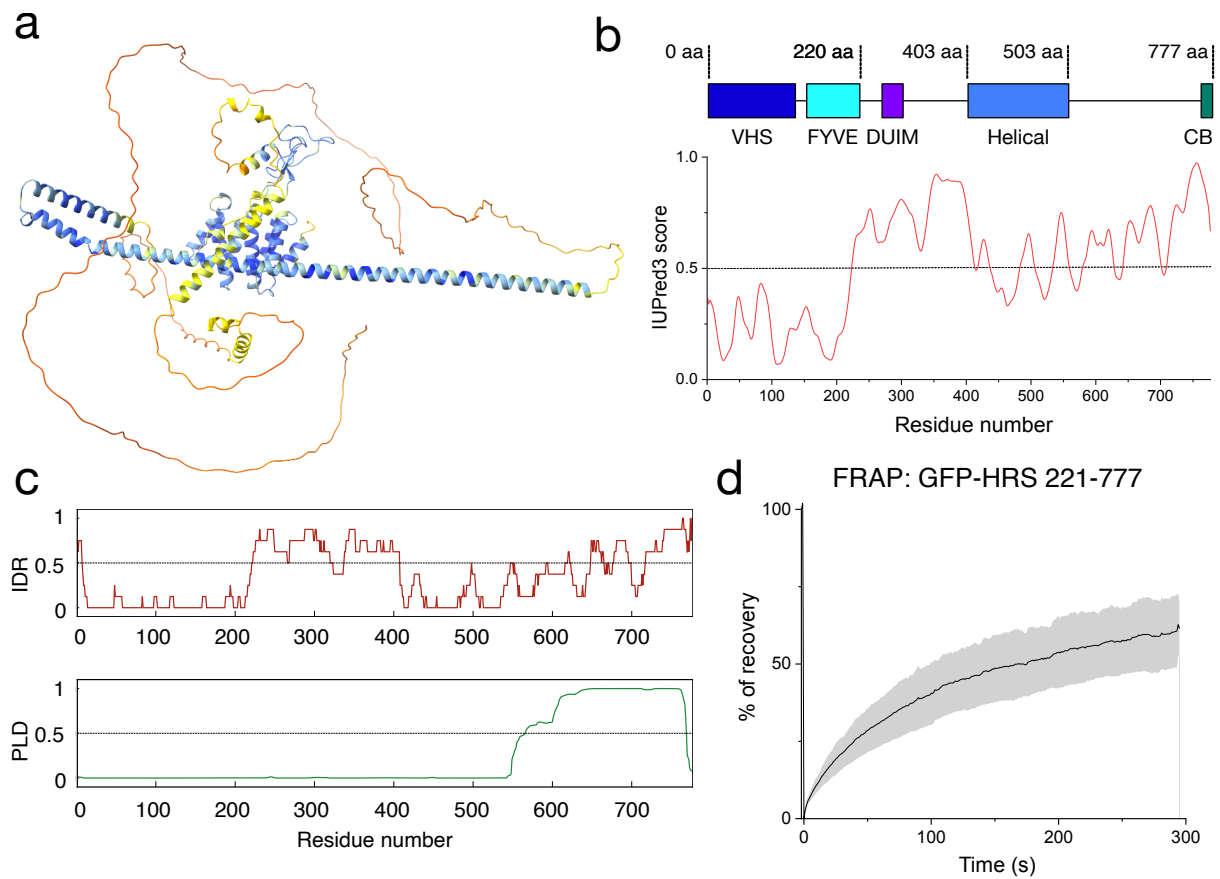
A scale bar is 10 μm . **d**, Representative time-lapse images of FRAP experiments of a 2 μM HRS (AlexaFluor-488 labeled) droplet on SLB membrane. The scale bar is 5 μm . **e**, 2 μM HRS (AlexaFluor-488 labeled, green) on SLB (magenta) containing 100% DOPC (left), 29% DOPC, 20% DOPE, 20% DOPS, 1% PI(3)P, and 30% cholesterol (middle), and 30% DOPC, 20% DOPE, 20% DOPS, and 30% cholesterol (right). All membranes are supplemented with 0.05% DOPE Atto-647n. The scale bar is 10 μm . The experiment was repeated three times.



Supplementary Figure 2

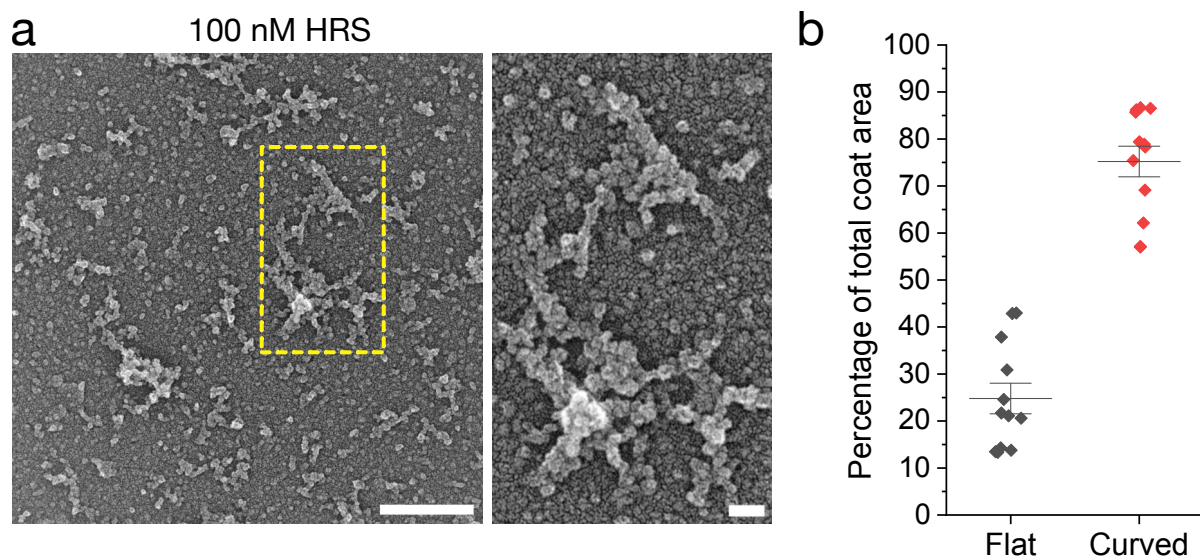
a, A schematic presentation of the CRISPR-Cas9 knock-in strategy (see Methods for details). **b**, Western blot of control HeLa cells and CRISPR-Cas9 mScarlet-I3-HRS knock-in cell lines. Different clonal lines are indicated with #1 and #4. **c**, Representative fluorescence microscopy

images of mScarlet-I3-HRS(#4) (magenta) cell lines with 5 min, 15 min, 30 min, or 45 min pulse of EGF-AlexaFluor-647 (green). Scale bars are 10 μm . **d**, Mander's colocalization coefficients of EGF-AlexaFluor-647 colocalizing with HRS. **e**, Representative confocal microscopy images of purified Scarlet-I3-HRS (0 nM, 50 nM, 100 nM, 200 nM) diluted with FluoroBrite media. A scatter plot of Scarlet-I3-HRS protein fluorescence over protein concentration. Data are from five experiments with a linear fit (red line) shown. **f**, Representative images of HeLa MZ Scarlet-I3-HRS(#4) cell lines imaged with the same settings as Scarlet-I3-HRS in panel e. A concentration of cytoplasmic Scarlet-I3-HRS was calculated based on fluorescence values measured from the cytoplasmic pool of Scarlet-I3-HRS and compared to a line fit in panel e. A box plot with median value, 25th and 75th percentiles, and the data range shown. The experiment was repeated two times. **g**, Expression levels of endogenous HRS and tagged Scarlet-I3-HRS in HeLa MZ cells and clonal cell line #4 were measured with Western blot and normalized with anti-tubulin staining as a loading control. The experiment was repeated three times.



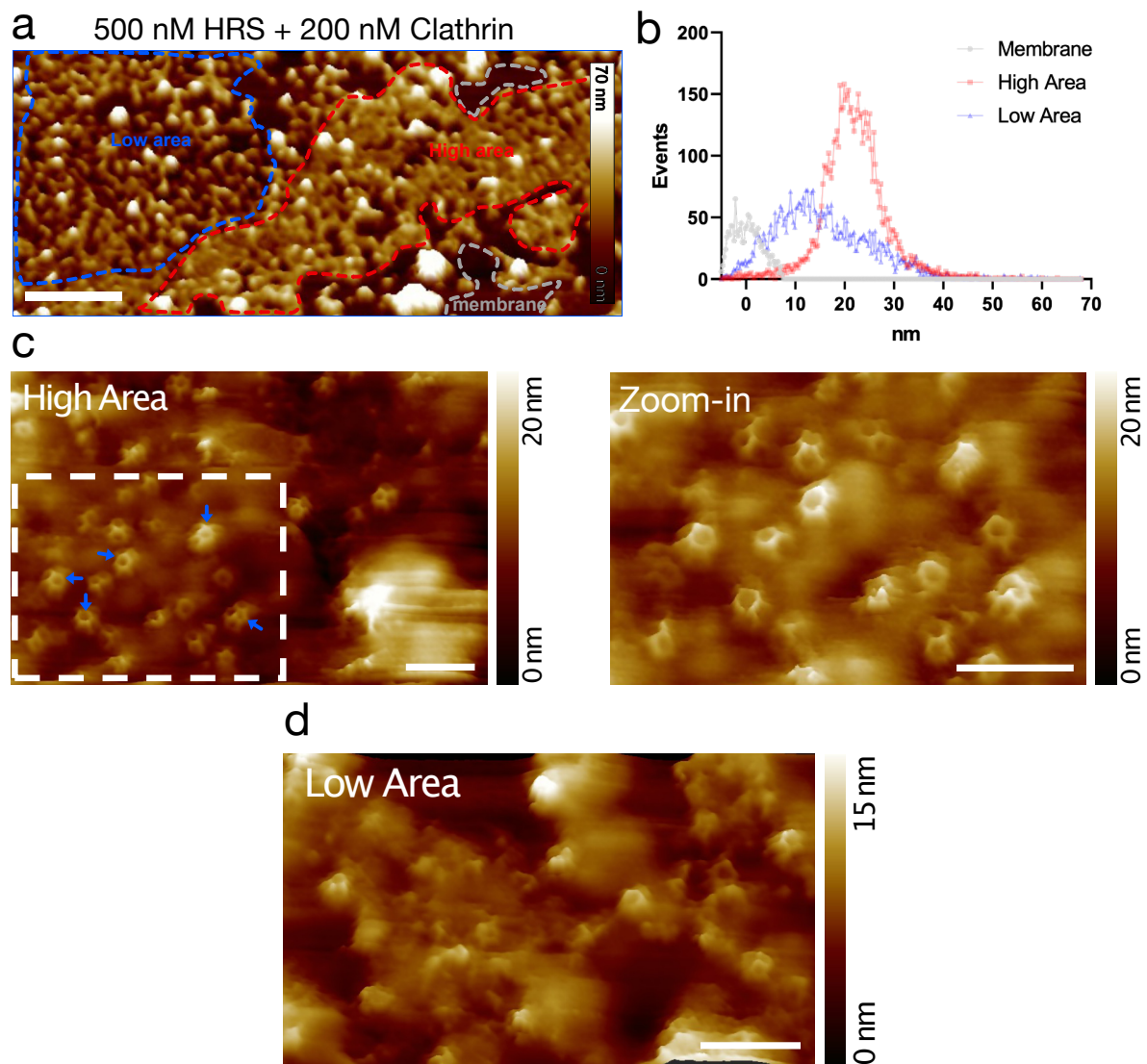
Supplementary Figure 3

a, An AlphaFold3 prediction of human HRS structure. **b**, Human HRS domain structure and IUPred3 prediction of unstructured regions in HRS protein. **c**, A MolPhase prediction of intrinsically disordered regions (IDR) and prion-like domains (PLD) in human HRS. **d**, FRAP of GFP-HR221-777 transiently expressed in HeLa cells. The data represent a mean of 20 measurements with a standard deviation shown.



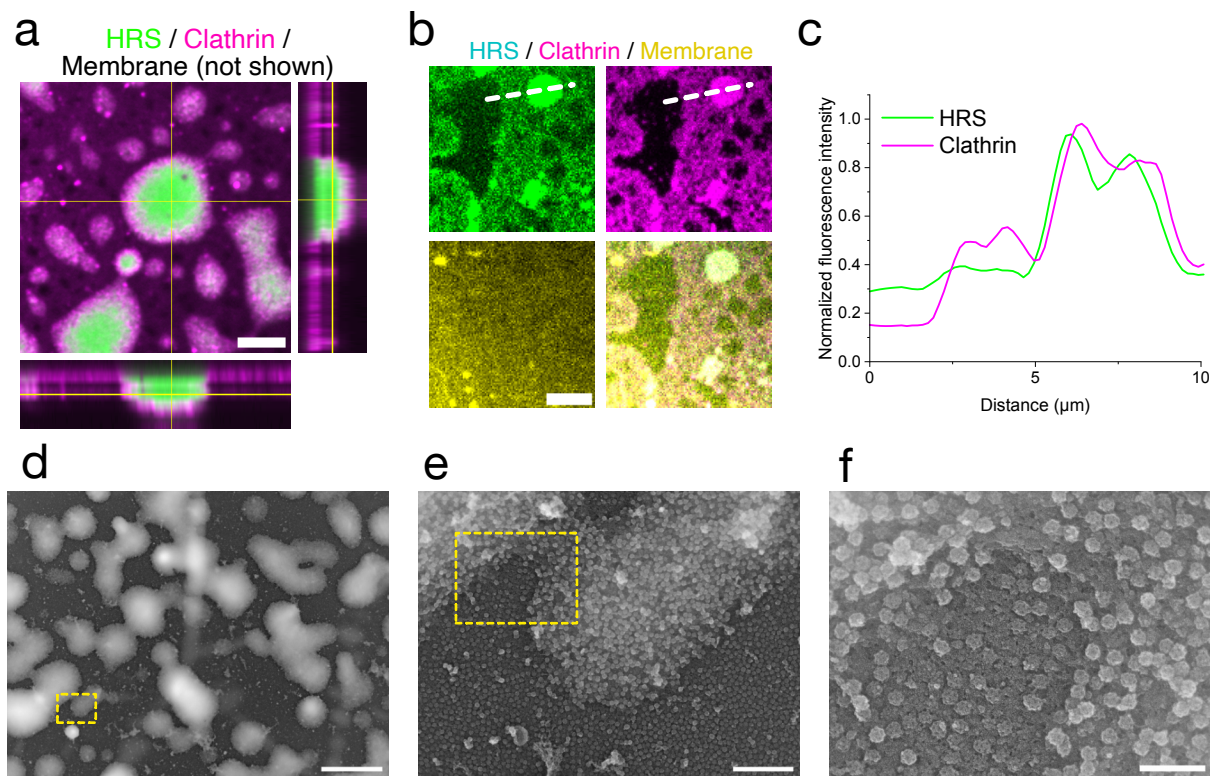
Supplementary Figure 4

a, A representative PREM image of 100 nM HRS reconstituted on SLB. Scale bar is 300 nm for the larger image and 50 nm for the zoom-in. **b**, The percentage of flat and curved clathrin coats in PREM samples reconstituted with 500 nM HRS and 200 nM clathrin. Each data point represents an individual image from two different sample replicates.



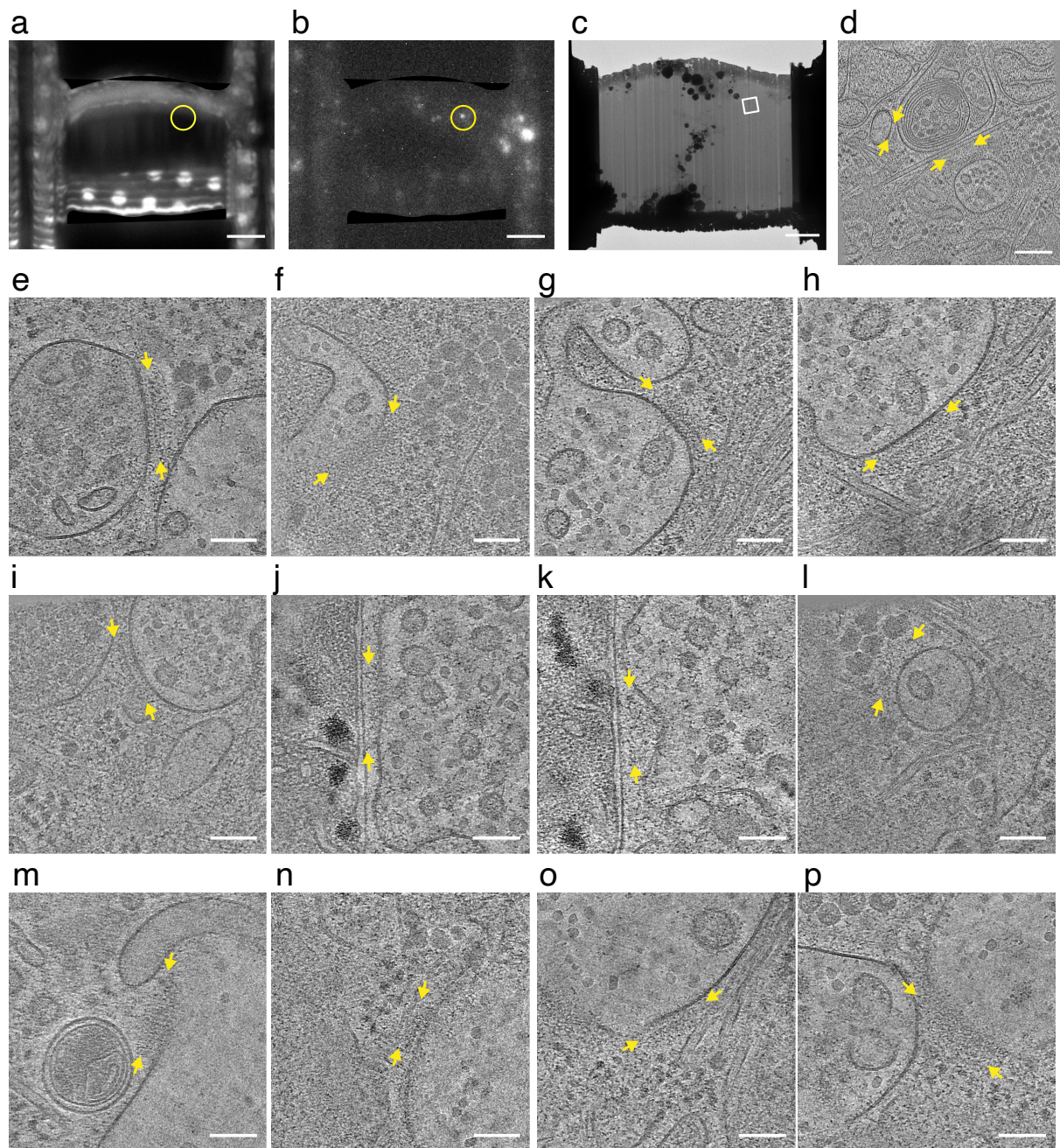
Supplementary Figure 5

a, A representative HS-AFM image of HRS-clathrin coat on SLB. The scale bar is 1 μm . **b**, A histogram of coat thickness. Thickness per pixel was measured from the regions indicated with respective colors in the HS-AFM image in panel **a**. **c**, A high-resolution image of the clathrin coat at the thick coat region (high area). The scattered box indicates the region in the zoom-in image. Scale bars are 200 nm. Blue arrows indicate recognized hexagons. **d**, A high-resolution image of the clathrin coat on the thin coat region (low area). A scale bar is 200 nm.



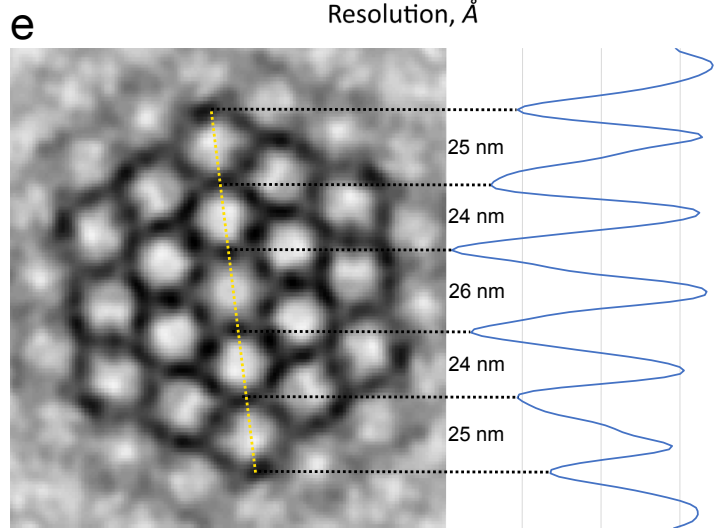
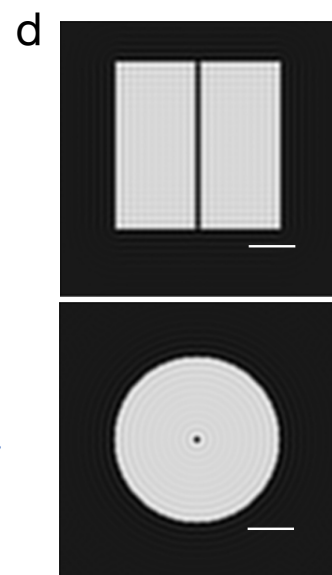
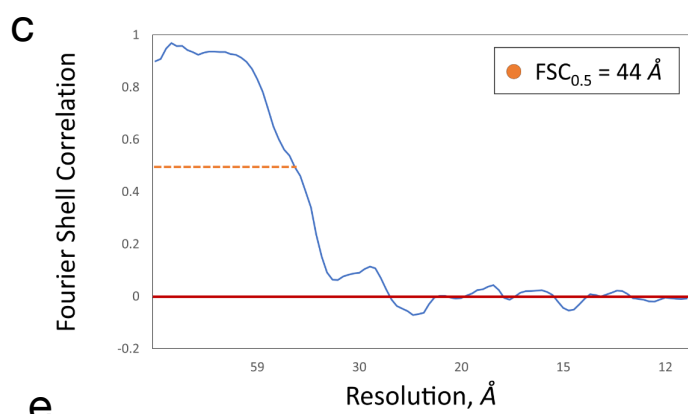
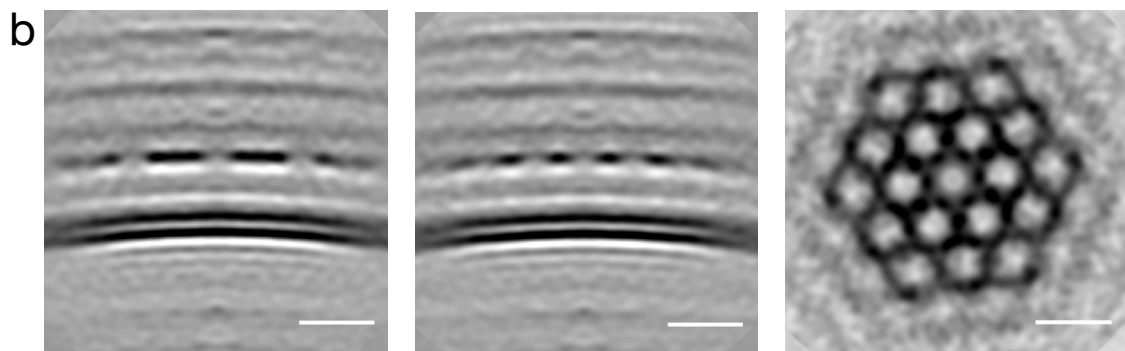
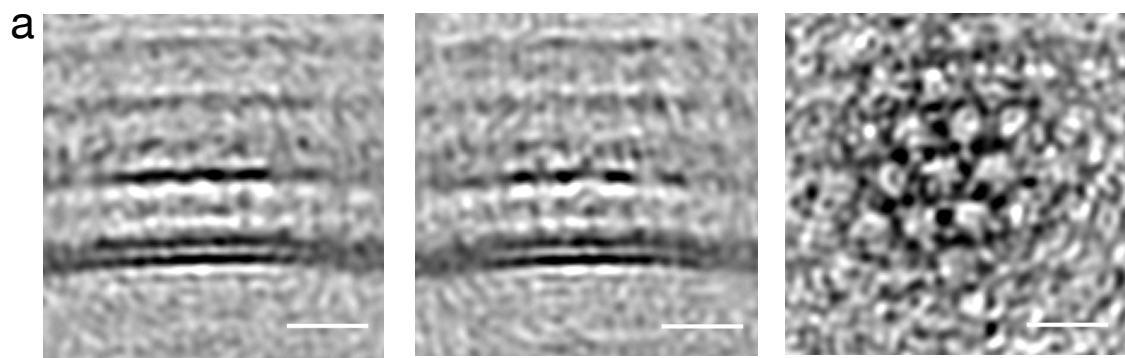
Supplementary Figure 6

a, A representative fluorescence microscopy image with orthogonal views of 2 μM HRS (AlexaFluor-488 labeled, green) and 200 nM clathrin (AlexaFluor-568 labeled, magenta) on SLB. The scale bar is 5 μm . **b**, Representative fluorescence microscopy images of 2 μM HRS (AlexaFluor-488 labeled, green) and 200 nM clathrin (AlexaFluor-568 labeled, magenta) on SLB (yellow), forming clathrin-coated two-dimensional condensates. LD=low-density HRS, HD=high-density HRS, D=droplet condensate. The scale bar is 10 μm . **c**, Line profile analysis of AlexaFluor-488 labeled HRS and AlexaFluor-568 labeled clathrin over three HRS populations on supported bilayers in panel b. **d-f**, PREM images of clathrin-coated spherical HRS condensates reconstituted with 2 μM HRS and 200 nM clathrin. **g-h**, PREM images of clathrin-coated two-dimensional HRS condensates reconstituted with 2 μM HRS and 200 nM clathrin. Scale bars are 10 μm (d), 1 μm (e) and 300 nm (f-h).



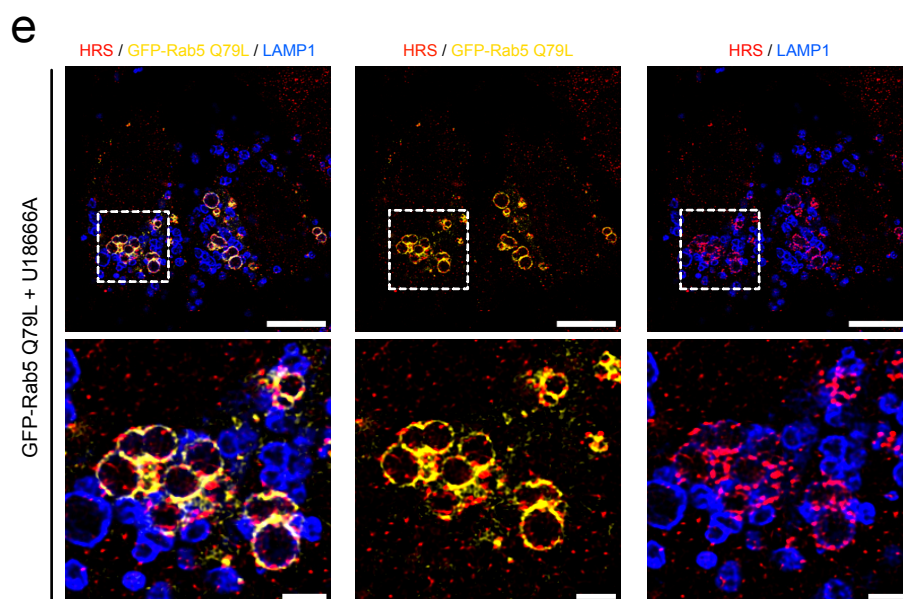
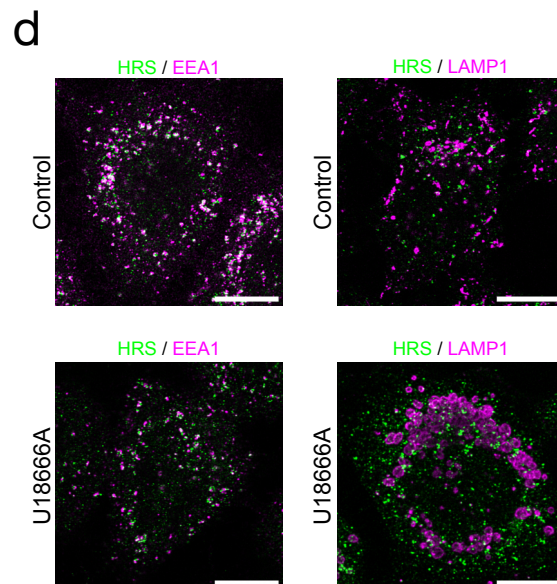
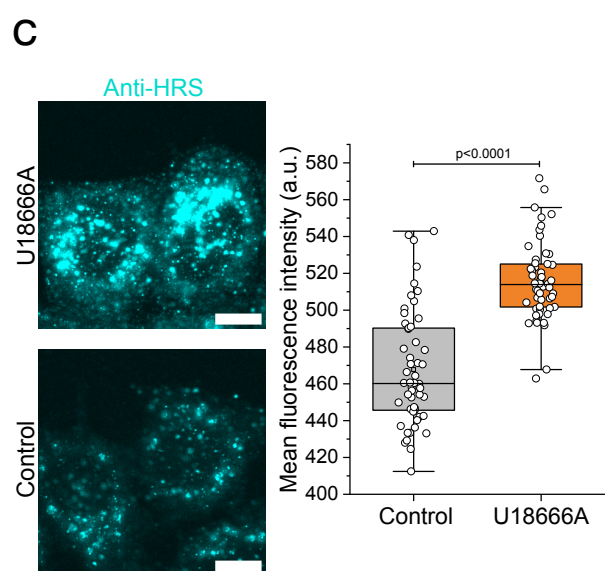
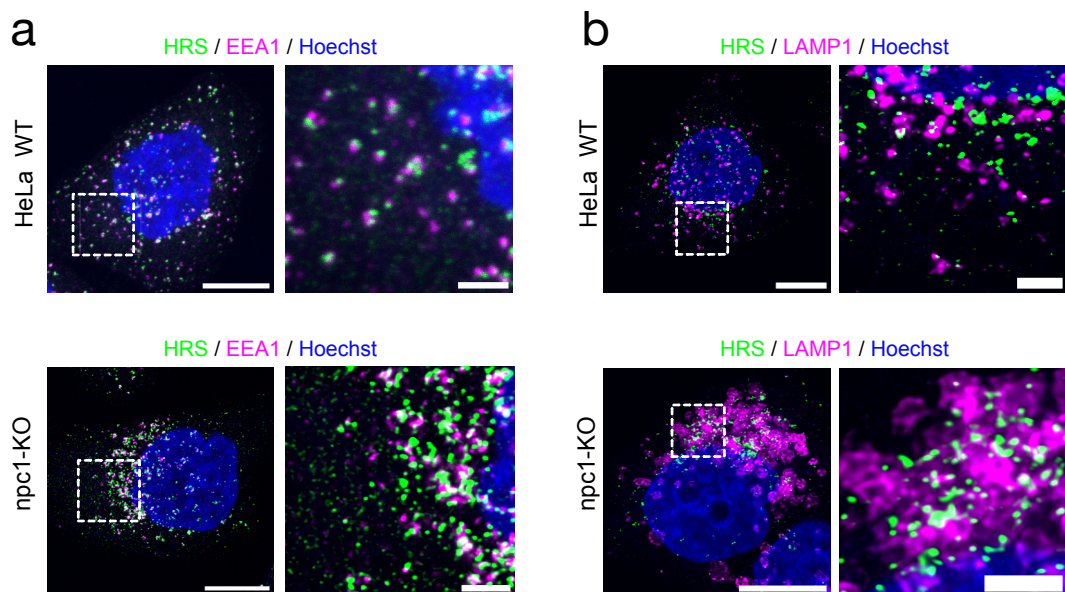
Supplementary Figure 7

a-d, The cryo-CLEM workflow for the acquisition of the data shown in Figure 6a-c. **a**, An image of the lamella after rough milling, acquired by iFLM in reflection mode. **b**, A fluorescence image of the same lamella acquired by iFLM in fluorescence mode, showing the signal of AlexaFluor-647 EGF. The yellow circle indicates the target signal. **c**, A cryo-EM overview of the final lamella. The white square indicates the area of acquisition of the tomogram shown in panel d and Figure 6a. **d**, A virtual slice through the tomogram acquired as indicated in panel c. Note that this slice corresponds to a different z-position than the one shown in Figure 6a, and is rotated by 180° relative to Figure 6a. **e-p**, All regions with a putative protein coat on endosomes (yellow arrows) identified in tomograms acquired similarly as indicated by the workflow in panels a-d. Scale bars are 5 μm (a-b), 3 μm (c), 500 nm (d), and 100 nm (e-p).



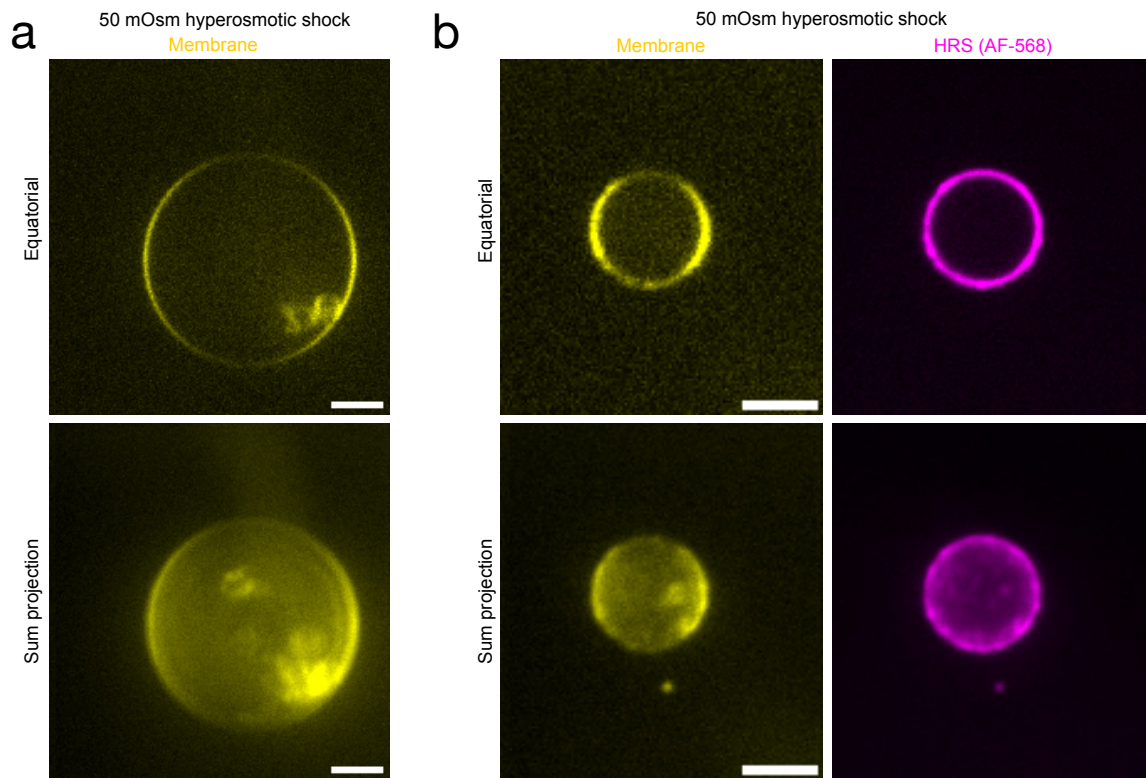
Supplementary Figure 8

a, Side views (XZ and YZ) and top view of the initial structure, obtained using the final 440 subvolumes without symmetry applied. Scale bars are 20 nm for side view images and 40 nm for top-view images. **b**, Side views (XZ and YZ) and top view of the refined structure, obtained using the final 440 subvolumes, the mask shown in **d** and with C6 symmetry applied. Scale bars are 20 nm for side view images and 40 nm for top view images. **c**, A Fourier Shell Correlation (FSC) graph. **d**, The mask used for the refinement. **e**, The analysis of vertex-to-vertex distance of hexagonal lattice.



Supplementary Figure 9

a, A representative fluorescence microscopy image of HeLa wild type and NPC1 knockout cells stained with HRS (green) and EEA1 (magenta) antibodies. **b**, A representative fluorescence microscopy image of HeLa wild type and NPC1 knockout cells stained with HRS (green) and LAMP1 (magenta) antibodies. In panels a-b, scale bars are 5 μm for larger images and 2 μm for zoom-in images. Experiment was repeated three times. **c**, Representative fluorescence microscopy images of HeLa control cells and cells treated with U18666A stained with HRS antibody (cyan). Scale bars are 10 μm . Mean HRS fluorescence intensity in U18666A treated and control HeLa cells. Experiments was repeated three times. **d**, Representative images of control HeLa cells and U18666A drug-treated cells with HRS, EEA1, and LAMP1 immunofluorescence staining. Scale bars are 20 μm . **e**, A representative fluorescence microscopy image of HeLa cells transfected with GFP-Rab5 Q79L mutant (yellow) and treated with U18666A, and stained HRS (red) and LAMP1 (blue) antibodies. Scale bars are 5 μm for larger images and 2 μm for zoom-in images. The statistical test in panel c is the Welch's t-test.



Supplementary Figure 10

a, GUV (yellow) containing 30% cholesterol under hyperosmotic shock. The equatorial plane and sum projection are shown. **b**, GUV (yellow) containing 30% cholesterol incubated with 500 nM HRS (AlexaFluor-568 labeled, magenta) under hyperosmotic shock. The equatorial plane and sum projection are shown. Scale bars are 5 μm .

Supplementary Tables

Supplementary Table 1. Lipids used in this study. Their abbreviation, full name, commercial source and catalog numbers are mentioned.

Abbreviation	Full name	Source	Catalog #
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine	Avanti Polar Lipids	850375
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine	Avanti Polar Lipids	850725
DOPS	1,2-dioleoyl-sn-glycero-3-phospho-L-serine	Avanti Polar Lipids	840035
PI(3)P	1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3'-phosphate	Avanti Polar Lipids	850150
Cholesterol	cholesterol (plant)	Avanti Polar Lipids	700100
TopFluor-cholesterol	23-(dipyrrometheneboron difluoride)-24-norcholesterol	Avanti Polar Lipids	810255
DOPE Atto-647n	Atto 647n 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine	Attotec	AD 647N-161

Supplementary Table 2. Lipid compositions used in this study. Lipid mix number as referred in text, key features, and full composition with molar percentages are listed.

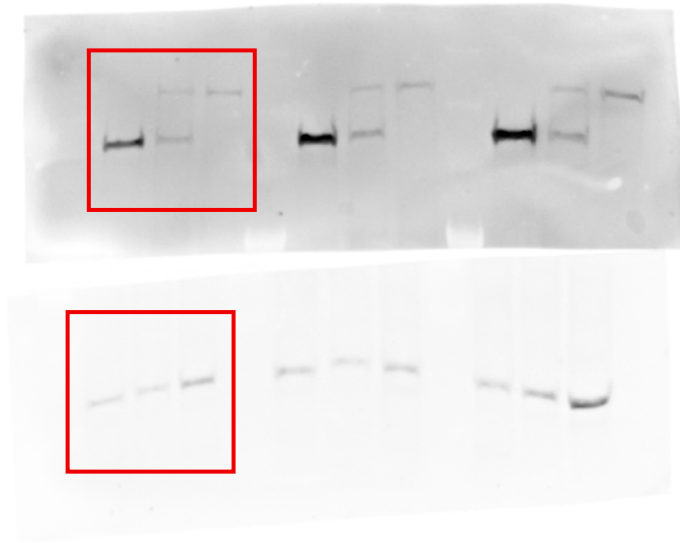
Lipid mix #	Key features	Full composition	Mol%
# 1	1% PI(3)P / 15% cholesterol	DOPC:DOPE:DOPS:PI(3)P:cholesterol:DOPE Atto-647n	44:20:20:1:15:0.05
# 2	0% PI(3)P, 20% DOPS	DOPC:DOPE:DOPS:cholesterol:DOPE Atto-647n	45:20:20:15:0.05
# 3	1% PI(3)P / 0% cholesterol	DOPC:DOPE:DOPS:PI(3)P: DOPE Atto-647n	56:20:20:1:0.05
# 4	1% PI(3)P / 30% cholesterol	DOPC:DOPE:DOPS:PI(3)P:cholesterol:DOPE Atto-647n	29:20:20:1:30:0.05
# 5	1% PI(3)P / 30% cholesterol / 1% TF-cholesterol	DOPC:DOPE:DOPS:PI(3)P:cholesterol:TopFluor-cholesterol:DOPE Atto-647n	29:20:20:1:29:1:0.05
# 6	1% PI(3)P / 30% cholesterol / 0.1% TF-cholesterol	DOPC:DOPE:DOPS:PI(3)P:cholesterol:TopFluor-cholesterol:DOPE Atto-647n	29:20:20:1:30:0.1:0.05
# 7	100% DOPC	DOPC:DOPE Atto-647n	100:0.05

Supplementary Table 3 Data collection and processing statistics for the StA

Microscope	Titan Krios G4
Magnification	42,000
Voltage (kV)	300
Cs (mm)	2.7
Total electron dose ($e^-/\text{\AA}^2$)	≤ 120
Defocus range (μm)	3.5 – 4.1 (determined with ctfplotter)
Camera	Falcon 4 with Selectris energy filter
Pixel size (\AA)	2.97 (data collection) 5.94 (processing)
Number of tomograms	8
Symmetry imposed	C6
Initial number of particles	1809
Final number of particles	440
Refinement method	Non-independent half-sets
Map resolution (\AA)	44
FSC threshold	0.5

Supplementary Table 4. Antibodies and dilutions used in immunofluorescence imaging (IF) and in Western blot (IB) in this study.

Target	Antibody name	Source	Catalog number	Host species	Dilution in IF	Dilution in WB
HRS	Anti-HGS	Abcam	Ab72053	Rabbit	1/300	1/1000
EEA1	Anti-EEA1	BD Biosciences	610457	Mouse	1/300	-
LBPA	LBPA antibody serum	Jean Gruenberg lab, University of Geneva	-	Mouse	1/100	-
LAMP1	Anti-LAMP1 (H4A3)	Developmental Studies Hybridoma Bank (DSHB)	H4A3	Mouse	1/500	-
Actin	Anti-actin	Sigma-Aldrich	A2066	Rabbit	-	1/1000



Original Western blots of Scarlet-HRS cell lines in Extended data figure 2b. Blots cropped for the figure are indicated with red boxes. The upper blot is an anti-HRS, and the lower is an anti-actin blot. Three independent samples of three different cell lines were blotted simultaneously.