

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

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**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, Hakala et al. report reconstitution of membrane-associated ESCRT-clathrin complexes, which in cells serve as endosomal sorting platforms that recruit ubiquitinated cargo for sorting into intraluminal vesicles. Given insufficient understanding of the structure and kinetics of these platforms, this study addresses an important question. The reconstitution system developed here represents a supported lipid bilayer containing PI3P and cholesterol, which is sequentially incubated with the ESCRT-0 protein HRS and clathrin. To characterize these *in vitro* complexes, as well as their counterparts in cells, the authors use an impressive set of cutting-edge techniques and present a series of interesting observations. They are: (1) the formation of HRS aggregates in solution and their spreading on the lipid bilayer; (2) the formation of unusual clathrin assemblies on the HRS-coated membranes; (3) the three-layered structure of endosome-associated platforms in cells, in which the endosome-proximal layer is made by clathrin; (4) an important role of cholesterol in the assembly of HRS-clathrin structures that suggests an existence of a feedback between the cholesterol-mediated HRS recruitment and HRS-mediated cholesterol immobilization; and (5) membrane-flattening ability of the GUV-associated HRS-clathrin assemblies in conditions of reduced membrane tension.

Besides these positive aspects of the study, there are also major problems. First, there is a serious mismatch between some data and the way the authors describe and interpret them, as explained in detail below. As a result, the major conclusions of this study are poorly supported. Second, the authors make little effort to mechanistically interrogate their findings. For example, some mechanistic insights into peculiar HRS behavior might be obtained by testing roles of individual HRS domains.

The specific concerns are presented below.

1. One main conclusion of the study is that HRS undergoes phase separation and forms liquid condensates. If it was so, these “condensates” would undergo fast turnover in the FRAP assay, but they poorly recovered. Also, phase separation indicates homogeneous-to-clustered redistribution, whereas HRS behaves in an opposite way: HRS clusters spread out into a more homogenous layer. However, there is a conflict here between the data obtained fluorescence microscopy (Figure 2A and video 3), where HRS spreads out, and the HS-AFM data (Video 4), where HRS clusters just appear from nowhere as if falling down from above. This discrepancy needs to be explained. Thus, the presented data show that rather than forming liquid condensates, HRS instead forms solid aggregates, probably, through some kind of strong self-association.

2. Another main conclusion in the study is that after binding to the HRS-coated lipid bilayers clathrin forms flat lattices. In fact, the most striking feature of the shown EM images, and the associated video, is that clathrin almost invariably forms small uniform spherical or hemi-spherical structures, which are tightly packed next to each other. Nowhere can one see extended flat clathrin lattices with a continuous hexagonal pattern, as it should be for flat clathrin lattices. It is true that the areas outside these collections of round clathrin cages have a rough appearance, which is likely explained by the presence of HRS. The authors need to show the structure of the HRS coat in the absence of clathrin. Even without this control, it can be inferred from the AFM images in Figure 2D that HRS by itself forms a rough surface, consistent with its ability to form aggregates. The small spherical clathrin structures shown by the authors strongly resemble the empty cages of ~80 nm in diameter, which clathrin forms in solution by itself. It seems that such cages are formed here, whereas HRS links them to the membrane and somehow to each other, often as a monolayer of cages. It would strengthen the study if the authors figured out how HRS does so, for example, using various HRS mutants.

3. Yet another main conclusion of the manuscript is about multilayering of the HRS-clathrin complexes. However, this point is unclear and rather ambiguous. From platinum replicas (Extended figure 2), it seems that by multilayering the authors mean that the flat layers of small clathrin cages sit on top of each other, in which case the thickness of a single layer should be similar to the cage diameter (~80 nm). However, this raises the question of whether HRS is present in the upper layers, as the authors propose multilayering of the HRS-clathrin complexes, but the provided images are inconclusive on this point. More importantly, the authors find by AFM (Figure 3d,e and extended figure 5) that the thickness of a layer is ~10 nm. What is this layer then? In fact, the AFM images do not give an impression of layering, but rather of a very rough landscape. Also, the quantitative results showing distribution of sample heights poorly match the images. According to the colored scale bar in Figure 3D, the white peaks, which are quite numerous, should be ~100 nm high, but such numbers are not seen in the graph in Figure 3E. Also, the authors do not provide details of how the heights were measured. What kind of regions of interest were used? How were they selected? How many of them were quantified? How do the statistics on these measurements look like? Given that so many peaks and valleys are seen in the image, it is hard to imagine that the difference between 10 and 20 nm is significant.

4. It is not clear what exactly is similar between the reconstituted HRS-clathrin coats and endosomal platforms observed by cryoEM, as stated on p. 10, ll. 283-285. The authors found only one clathrin layer in the endosomal platforms in cells, as compared to what they claim as multilayering in vitro. The appearance and dimensions of these two types of structures also look hardly comparable. The only similarity is that clathrin forms hexagonal lattices, which is hardly a surprise.

5. Quantification methods and statistical analyses for many sets of data are not provided.

Minor comments:

1. p. 6, ll. 176-177: "Notably, these protein patches did not show high-ordered structure, which indicates they form through phase separation". There is no logic in this statement. A lack of ordered structure, like in any kind of mess, can occur without phase separation.

2. p. 7, ll. 193-194: References cited here report true liquid-like condensates, which exhibit fast recovery and phase separation, and therefore they do not resemble the solid-like structures made by HRS, as stated by authors.

3. Figure 5F: Clathrin staining after npc1 KO needs to be shown. The authors also need to explain how they distinguished endosome-associated clathrin and plasma membrane-associated clathrin for quantification purposes, as well as provide other quantification details.

4. p. 13, ll. 404-408: The references on line 408 do not report the multilayering of clathrin on endosomes, as the authors claim in this sentence, except to some extent for the paper by Sachse et al. However, Sachse et al. report a "bilayered" structure of clathrin-containing platforms, but do not claim that both layers are made of clathrin. Instead, it is clear from the images that the two layers are different – the membrane-proximal dense layer is not a clathrin coat and might contain HRS, while the membrane distal layer is more transparent and resembles typical clathrin coats.

5. If authors intend to make their images accessible to color-blind readers, they should change the magenta+cyan combination to either cyan+red or magenta+green. Given that magenta is red+blue and cyan is green+blue, the readers still need to be able to distinguish red and green.

6. Extended Figure 3a: Spell out the HRS domains and explain what they do.

7. Extended Figure 4b,c: Flip either panel B or C to align the line on B with the linescan in C.

In summary, the authors present several intriguing observations obtained by technically powerful approaches but often provide misleading interpretations of their findings and, in most cases, do not achieve informative mechanistic insights into these phenomena. The information about quantitative approaches is largely missing. The study can be strengthened by an adequate description of the results and their proper discussion that should be consistent with the data, and by additional experiments that can provide at least some mechanistic insights into observed phenomena.

Reviewer #2

(Remarks to the Author)

This manuscript from the Roux and Kaksonen labs presents several pieces of very interesting experimental data which explore the ability of HRS proteins to form condensates, recruitment and assembly of clathrin at the surfaces of these condensates, the possible role of cholesterol and cargo in the process, and the possible implications of these observations for endosomal clathrin assembly and endosomal cargo sorting. My overall impression is that these are high quality and significant data that should be published in a visible venue such as Nat Comms. However, I feel the authors did themselves a disservice by presenting their data in a convoluted and confusing manner. I think the paper will appeal to a broad audience much better if it is reorganized and potentially shortened. Below I offer some suggestions to hopefully help the authors in this process and make the most of their findings.

1. Early in the paper the authors should explain clearly what they mean by the "unique bi-layered organization" of HRS-

clathrin. The description of the multi-layer structures is not quite clear. Are there multiple layers of clathrin or of hrs or of both? The authors refer the reader to the videos but these too are confusing. It would be helpful if the authors could explain in greater detail how they are interpreting the images/video and perhaps quantify some aspect of them to show that a multi layer structures is indeed present. I finally understood it (I think) after taking a close look at extended data Fig. 2. The authors should describe this figure in detail in the main text as well as the quantification they performed there, none of which are mentioned at all in the text. The authors should consider moving this figure (or at least parts of it) to the main text to make this critical point clearer, earlier in the paper.

2. Similarly, the description of the multi-layered protein structure on endosomes is interesting but rather confusing. The authors identify fairly convincingly that one of the layers is a clathrin lattice. What is in the other layers? Is it HRS? Are there other key proteins? It is not so clear to me that the results here are similar to their in vitro findings, where there were multiple layers of clathrin, perhaps interlaced with HRS. The authors need to more precisely justify their claim of agreement between in vitro and live cell results.

3. Very importantly, the authors should explain the biochemistry of HRS interactions with clathrin at the beginning of the paper. In particular, what is the domain architecture of HRS and does it contain known binding motifs or domains for clathrin? I looked it up in the literature and found HRS has a well-established clathrin binding box. Astoundingly, this was never mentioned in the paper. Has HR been previously shown to assemble clathrin in vitro?

4. Also, what about the domain architecture of HRS might explain the authors' finding that HRS forms condensates? Not all disordered proteins form condensates. What is it about the amino acid composition of the IDRs perhaps that suggests phase separation? Have the authors examined the sequence using LLPS prediction software of which there are now many freely available?

5. The authors point out that their HRS-clathrin structures are flat in sharp contrast to earlier work with endocytic adaptors. It would be more convincing if at least one of these studies were repeated side by side with the current work to ensure that it is truly a difference in the proteins rather than a difference in the membrane preparation or the many other parameters that go into the preparation and imaging of these complicated samples. Some of the cited studies come from the same labs as the authors so it should be feasible. As ~10 years have passed and the lab members/materials/protocols are now likely changed, it seems a stretch to make a direct comparison with past data without repeating some part of it as a control.

6. To truly demonstrate that HRS can condense in 2 dimensions on the membrane, rather than falling down from solution, the authors should dramatically lower the protein concentration (10 to 100 fold) such that 3D protein condensates never form and then allow the membrane to become saturated in 2D with protein and spontaneously undergo phase separation, rather than simple wetting of the membrane by a 3D condensate. Previous work from several labs has shown that the saturation concentration on a 2D surface is typically 10-100X below that of 3D solution, owing to the reduced dimensionality. This is an important experiment, as the concentration of HRS in cells is likely too low for assembly of 3D condensates in the cytosol that can wet membranes.

7. The authors state "Notably, these protein patches did not show high-ordered structure, which indicates they form through phase separation (Figure 2d)." This sentence should be amended or eliminated. The observation that a material lacks a high degree of order is not sufficient to prove how it formed through the process of phase separation.

8. The sections of the paper on cholesterol feel tangential, as they are not well integrated into the rest of the story. It is not clear why they were included in this paper. I suggest saving them for a future study, as they seem to represent a separate topic that should be more fully explored on its own. The paper has plenty of data without these studies to warrant publication.

9. The abstract and introduction focus almost exclusively on flat clathrin lattices, while the authors' images show mainly curved lattices. I do not understand why the authors prefer to focus their interpretations on the flat lattices, which appear overall less apparent in the images.

10. I could not make much sense out of the final cartoon in Figure 7d. Why is the membrane budding? This is never clearly explained. What role is clathrin supposed to have in budding in the opposite direction of clathrin's curvature preference? This was never mentioned. I am sorry to be obtuse, but I got totally lost in trying to interpret this cartoon. A much clearer summary that is better grounded in the direct observations of the authors is needed.

#### Minor points:

1. The arrangement of the first section of the paper is confusing. The authors start out with a complex system of membranes, HRS and clathrin. Then they go back to a simpler system of just HRS and membranes, then back again to the full system. The membrane composition is also changed several times in ways that are not very clear. For example, the composition of the first experiment is listed but then it simply says that 1% pi3p was used, which is the same as at first, so it is not clear what was changed. This is hard for the reader to follow. It would be easier to start with the simplest experiment and work up to the more complex experiment.

2. Various features of HRS (five domain, disordered regions) are mentioned at various places throughout the manuscript. It would be helpful to present the domain architecture of HRS from N to C in one place near the beginning of the paper. Doing so would increase the clarity of the story.

3. The statement "cholesterol promotes membrane bending during CME" is perhaps too strong, as most studies are either correlative experiments in cells or highly simplified in vitro studies. Perhaps say instead "cholesterol correlates with membrane bending during CME"

4. The authors comment that clathrin recruitment to membranes did not saturate at high HRS concentrations. How do the authors rationalize this observation? What is its significance?

(Remarks to the Author)

This work provides an interesting and convincing demonstration of a new mechanism for the formation of flat clathrin lattices through an elegant combination of reconstitution experiments and in situ imaging of endosomes by cryo-tomograph. The main findings are that the ESCRT-0 protein HRS forms condensates on membranes that promote clathrin assembly and that cholesterol plays an important regulatory role. This work provides new mechanistic insight in the function of ESCRT-0 in sorting ubiquitylated cargoes and it also provides an intriguing new example for a physiological role of protein condensates. The manuscript is well-written and the conclusions are largely supported by the data. The authors should address a few points for completeness and clarity.

- 1) In the HRS condensate experiments the authors use labeled HRS; they should show evidence regarding the question whether or not the label influences the HRS phase behavior.
- 2) In their SLB assays, the authors sequentially incubate the SLBs with HRS and clathrin with an intermediate washing step. They should document and discuss the stability of HRS-membrane binding.
- 3) The description of the platinum replica EM data (Fig 1f) is very qualitative with descriptions of "frequent large patches", "often observed round clathrin islands" and "cup-shaped structures with various degrees of curvature". The authors should make these descriptions more precise and where possible quantitative.
- 4) The authors should discuss in more detail the physiological relevance of their reconstitution assays. Specifically, they should motivate their choice of lipid composition and the protein concentrations and discuss the implications of combining human HRS and bovine clathrin.
- 5) The viscosity of the HRS films should be more carefully interpreted. The authors show FRAP data that suggest that recovery is slow. Did they check that the SLBs themselves are fluid? Does the fluidity of the HRS film change over time? Can they estimate a lower bound for the fluidity and is this consistent with the time scale (and ability) of the HRS droplets to fuse? Is the fluidity of membrane-wetting HRS films the same as the fluidity of bulk HRS droplets? Is the HRS film in fact fluid or a gel? In the discussion, the authors briefly speculate that the HRS-clathrin coat may be flat due to high rigidity of the HRS condensate. This should clarify this point and if possible investigate the mechanical properties of the film in more detail.
- 6) In measurements of cholesterol diffusion under HRS and HRS-clathrin coats, the authors report that cholesterol diffusion is more reduced under HRS-clathrin than under HRS alone. They should discuss why they think this is the case.
- 7) For the data on cargo cluster recruitment to GUVs (Fig 7) the authors should supply quantitative analysis of their claims that cargo clusters appear larger and more stable in presence of cholesterol.
- 8) the authors should clarify their statement on p18 line 564 "To avoid unwanted assembly of clathrin cages in solution, all experiments were performed at physiological pH and salt concentrations". Furthermore, they should specify the "microfluidic device" that they mention.
- 9) in Fig 1B: why is the signal in the membrane channel grainy and why does this graininess increase over time (and which lipid is labeled)? And in Fig 1D: how is the nucleation rate determined from the data in Fig 1C?
- 10) In Fig 2D: why is there a membrane island on the mica and what is the significance of this? And in Fig 2E: what is the meaning of "High area"? (similar in Extended Data fig 5: what are "high region and low coat regions"?)
- 11) Fig 3e: is this histogram for the image in Fig 3d? And how representative is fig 3d-e?
- 12) Fig 6c/D: why is the scaling of the intensities with cholesterol mol% different for HRS and clathrin?
- 13) Fig 7B: the authors should annotate these images and explain more clearly what can be concluded from these data

Reviewer #4

(Remarks to the Author)

In my opinion this manuscript is well-written, technically sound and contributes to the field of cellular and structural biology by providing new insight into mechanisms of vesicle budding driven by ESCRT. I admit I am not an expert in membrane remodeling or ESCRT but to me, it seems fine to publish this manuscript in Nature Communications. However, I have one major concern regarding the cryoET analysis and a few minor concerns with the figures. With regard to the cryoET, I feel like the analysis is lacking and should be addressed in the revised manuscript. Some of my points are minor and might be best addressed in the letter to the editor and reviewers.

Main Suggestions for improving the manuscript

Major points:

1. In the abstract "Correlative cryo-tomography of HRS-labeled endosomes revealed a pure hexagonal lattice, consistent with flat clathrin structures."

It's not obvious to me that the cryoET data shows this. When I read the abstract, I was expecting to see multiple hexagons in the tomograms but I did not.

Would it be possible to (1) segment the tomograms to show the different proteins and membranes, (2) use Isonet to denoise and fill in missing wedge information for the extracted subvolumes, and (3) superimpose the subvolume average into a tomogram using the XYZ coordinates of the subvolumes that were used to generate the final map (ie the IMOD command clone model). I think the segmentation and use of Isonet (or something similar) are common practices for cryoET aimed at characterizing unstructured cellular components.

2. I don't see any signs of the hexagon lattice in the tomographic data only the sub-volume averaging. Do you see this in the



tomographic data? If you see ordering can you modify the figures to show this? If you don't can you explain why? And clarify in the manuscript what you mean by a pure hexagon lattice.

I think that might be due to the orientation of the endosome membrane relative to the XYZ of the tomographic reconstruction. Can you confirm? What does it look like when you rotate the tomogram so we see the XZ or YZ tomographic slice? Or maybe show a selected area FFT in the tomographic slice to show ordering. I think this is an important point to consider for this paper given that the main conclusions are based on characterizing the structure organization of the ESCRT-0 in vivo and situ.

Minor points:

1. Figure 1. What happens if you add HRS to the membrane and image using PREM? It would be helpful for me to understand why this was not done. From reading the manuscript it seems like the authors could have done this experiment to gain a better understanding of how HSR organizes on a PM in the absence of clathrin. Maybe they have done this experiment in a previous publication and it's not necessary for this manuscript or it is common knowledge in the ESCRT field that I am not aware of. If that's the case can the authors address this point in the letter to the editor and reviewers?
2. Figure 1. I can see the clathrin cages in the PREM images but the flat clathrin patches seem disordered and do not have any signs of symmetry. Can the authors confirm? It's not obvious how closely the PREM images of the flat clathrin patches resemble the subvolume average about symmetry.
3. Figure 4. Is it possible to show an image or pseudo color or trace around the PREM images to indicate where the (1) low-density HRS, (2) two-dimensional HRS condensates, and (3) droplet-like HRS condensates are? The cartoon is helpful but just from looking at the PREM images, it is difficult for me to see where the boundaries of each are.
4. Figure 5. I don't see any hexagonal clathrin structures in the high-mag AFM images in Fig 5c-d. Can you confirm if there are any hexagons? And can you add an arrow over (maybe just one or two) the hexagons and the pentamers? I think the high mag insets should be larger so that it is easier for the reader to see the hexamer and pentamer. Even when looking at the pdf on my 27-inch computer monitor I have to zoom in and squint my eyes to see the pentamer and I don't see any hexagons.
5. It's not clear to me how well the fLM and cryoET data correlate. Can you modify Extended Data Figure 7 to show an fLM image next to a tomographic slice corresponding to panel d? It's difficult for me to tell which of the tomograms is associated with panels a-d and how well the fluorescent signal matches panels e-p concerning the yellow arrows pointing to the putative protein coat on the endosomes. In one of the CLEM papers, the authors reference Ganeva et al., 2023 do a fine job of showing CLEM. In this paper, the authors superimpose the fLM image with a tomographic slice (figure 1) to show how well the images correlate.
6. Extended Figure 8. Can you show an image of the subvolume average (440 subvolumes) with C1 symmetry? I appreciate the authors showing the initial subvolume with C1 but wonder how much the symmetry helps or how much of the appearance is due to imposing symmetry.
7. Figure 7d seems out-of-place in figure 7, can you make it a new figure?
8. Are you going to depositing the subvolume average to the EMDB?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The revised manuscript is significantly improved. The authors added new experiments with HRS deletion mutants and with low concentrations of HRS. They more adequately described gel-like properties of the HRS condensates, acknowledged the presence of clathrin cups and cages in the PREM images, as well as a lack of regular clathrin organization in the flat regions. They also showed the topology of the membrane coated with high concentrations of HRS (1  $\mu$ M for PREM and 500 nM for AFM), partially clarified the multilayering phenomenon, added statistics and addressed my previous minor comments.

Despite these improvements, I am still not convinced that AFM data are consistent with one of the main points of the manuscript that upon condensation HRS forms a 2D layer. In the rebuttal letter (although not in the manuscript), the authors explain the sudden appearance of HRS bumps in AFM images as events of fast HRS condensation when "the density of HRS onto the membrane passes the threshold to phase separation". If so, then HRS forms 3D aggregates instead of 2D layers. How is it consistent with the 2D layer hypothesis and with the data that suggest that HRS condensates are viscous and thus are unlikely to form instantly? The sudden disappearance of these 3D structures, as seen in video 3, also remains unexplained. In the newly added explanation of how the authors measured the heights of structures in AFM images, it is unclear what the "mask threshold" was and what it selected and rejected in these images.

It is also hard for me to share the authors' enthusiasm about flat clathrin-containing areas, as compared with those coated by clathrin cups and cages. One of the authors' main conclusions is that at low HRS concentrations clathrin mostly forms flat layers (lines 441-444). They also illustrate this point by the diagrams in Figures 10F and 4C. However, the PREM images show that virtually every "flat" clathrin island contains a cup or a cage. Although the authors acknowledge this fact in the main text, they do not take it into account in their final conclusions. Consistent with PREM data, the AFM images (Extended figure 4C) also show clathrin polygons only on the "hill tops", although authors describe them in the rebuttal letter as being "scattered onto the flat surface". Furthermore, since the "flat clathrin" areas, on which the authors focus their attention, are disorganized and do not show a clear polygonal pattern, it is inappropriate to refer to them as "coats" or "lattices", as the authors do throughout the manuscript, and even conclude the manuscript with the statement "Clathrin is assembled as a hexagonal lattice on HRS, which forms two-dimensional condensates on membranes" (lines 522-523). Together, these data suggest to me that, at least in this in vitro reconstitution system, the flat clathrin arrays are very unstable and form cups and cages as soon as they have enough neighbors. Therefore, the authors need to somehow strengthen their conclusion on this point or change it. It could be worthwhile to quantify the fractions of flat versus cup-covered regions on 2D HRS layers either as a function of time or concentration of HRS or clathrin to get a sense which type of the array is more common.

The point of multilayering is still not explicitly clarified, especially when it concerns the comparison of layers in vitro and in vivo. The newly added PREM data showing the structure of HRS layer alone suggest that HRS at high concentration can form at least two layers in vitro suggesting the possibility that a single layer of clathrin can be formed on top of the HRS "sandwich". In vivo, it seems that neither HRS nor clathrin form multiple layers, while additional layers seen in vivo are not relevant to what is observed in vitro. The clathrin arrangement is also different in these two conditions – flat hexagonal in vivo and unstructured flat or consisting of mostly pentagonal cups in vitro – the fact the authors also acknowledge. Thus, the only argument in favor of similarity between in vitro and in vivo layers is their similar thickness, which is not a strong argument. Another similarity – contribution of both HRS and clathrin – was already known before. Maybe I do not understand what authors mean by "multilayering". Is one layer of HRS plus one layer of clathrin considered as multiple layers? If so, the terminology needs to be explained. On the other hand, how then to deal with this point in relation to the data shown in figure 4?

Other comments:

1. Extended Data Fig. 2e needs a better legend or labeling. It is not clear what each panel shows.
2. In Figure 2a, it is not clear which areas show "homogeneous binding of HRS" and which represent naked membrane. They should be labeled. What is the interpretation of the patches in these images?
3. The text (lines 171-172) says that Figure 2b shows "the thickness of the HRS patches", whereas the legend to this figure says that it is "A histogram of HRS and HRS-clathrin coat heights". The label just says it is membrane and "high area". What is shown there?
4. A control image of membrane coated with 100 nM HRS alone is still missing to accompany data in figure 3f. It is needed in order to properly interpret the image with HRS and clathrin.
5. Video 7 does not play.
6. The text refers to "Figures 5 and Extended data Fig 5f-h, but the Extended data Fig 5 ends with panel f.
7. I do not understand the logic of the following sentence "without phase separation, the HRS-clathrin coat could grow indefinitely because it is flat." With phase separation it is also flat. I also do not see why non-flat coat cannot grow indefinitely.
8. Some Extended data figures remain in the cyan-magenta combination.

In summary, the authors present a large body of data, some of which are truly interesting and intriguing, but their main conclusions do not appear well supported by the data, and I still have hard time to formulate for myself what I have learned from this study. The data just do not come together too well to form a clear concept.

Reviewer #2

(Remarks to the Author)

The authors have been very thorough and thoughtful in their response to my questions as well as those of the other referees. I feel the paper is ready to be published. Congratulations to the authors on a fine work.

Reviewer #3

(Remarks to the Author)

The authors have satisfactorily addressed the reviewers' concerns leading to a greatly clarified manuscript.

Reviewer #4

(Remarks to the Author)

All my comments and suggestions were addressed in the revised manuscript. The revised manuscript looks good for publication.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I am generally satisfied with this revision. However, I suggest some editorial changes in order to improve clarity, correct typos and revise some statements that apparently have been inherited from the earlier versions of the manuscript and not edited in view of newer data.

1) p. 6, ll.131-132: "We noticed the spontaneous formation of HRS domains with the same intensity as the two dimensional condensate spreading out from droplets (Figure 1f, yellow arrowheads, Figure 1g)."

Please, clarify, what condition is being compared here to the spreading droplets. Although it can be deduced from the Figure 1g legend and subsequent text, it would be clearer to state it directly.

2) p. 7, l. 161-163: "...readily spread on the DOPS/PI(3)P-rich membrane as well as membranes containing 20% DOPS but no PI(3)P (Lipid mix 2, Supplementary Table 2), forming a dense protein film (Extended Data Fig. 1e)"

It should be mentioned here that an additional difference for the middle and right panels in Ext. Fig. 1e is the presence of cholesterol, which is not present in the left panel. In the light of the data shown later in the manuscript on the role of cholesterol, the interpretation of these data could be not so straightforward.

3) p. 7, l. 169: "... (Figure 2a, timepoint 39 min), which slowly separated into patches (Figure 2a, timepoint 64 min)."

The figure shows timepoints 40 min, 50 min, 61 min and 73 min, but not 39 min and 64 min.

4) p. 10, ll 286-287: "Finally, we observed multilayered clathrin coats on two-dimensional HRS condensates"

The authors rather showed single-layer clathrin coats (Figure 5 and Extended figure 6a-c) on multilayered 2D HRS condensates (Figure 4d).

5) p. 22, ll. 652-653: "Before and in between each protein addition, m"

This sentence seems truncated.

6) p. 47, l. 1383: "Scale bars in panel h..."

It should be panel a.

7) p. 48, l. 1396: "Representative PREM images clathrin coat reconstituted..."

Should, probably be "...images OF clathrin coat"

8) p. 48, ll. 1402-1403: "Two-dimensional HRS condensates lead to a predominantly flat clathrin coat."

Suggested revision: "Two-dimensional HRS condensates lead to a mixture of flat and dome-like clathrin coats." This is because the quantification newly included into the revision shows that dome-like coats represent ~80% of surface area and flat ones ~20% (Ext. Fig. 4b)

9) p. 50, l. 1462: "Sorting coefficient values were blotted..."

plotted?

10) p. 50, ll. 1488-1489: "Clathrin assembles on HRS condensate to form a multilayered protein coat, where clathrin coat is predominantly flat."

According to authors' data, clathrin forms a single layer (Figure 5 and Extended figure 6a-c) and is predominantly curved (80%, Ext. Fig. 4b). This statement needs to be revised accordingly.

11) p. 52, ll. 1541-1542: "Blue arrows indicate recognized hexagons."

These arrows are in panel c not panel a.

12) p. 52, ll. 1545-1546: "The scattered box indicates the region where the zoom-in image."

Revise the sentence.

13) p. 54, l. 1613: "...the gain and force applied to AFM tip for increased..."

'was' increased?

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Reviewer #1 (Remarks to the Author):

In this study, Hakala et al. report reconstitution of membrane-associated ESCRT-clathrin complexes, which in cells serve as endosomal sorting platforms that recruit ubiquitinated cargo for sorting into intraluminal vesicles. Given insufficient understanding of the structure and kinetics of these platforms, this study addresses an important question. The reconstitution system developed here represents a supported lipid bilayer containing PI3P and cholesterol, which is sequentially incubated with the ESCRT-0 protein HRS and clathrin. To characterize these in vitro complexes, as well as their counterparts in cells, the authors use an impressive set of cutting-edge techniques and present a series of interesting observations. They are: (1) the formation of HRS aggregates in solution and their spreading on the lipid bilayer; (2) the formation of unusual clathrin assemblies on the HRS-coated membranes; (3) the three-layered structure of endosome-associated platforms in cells, in which the endosome-proximal layer is made by clathrin; (4) an important role of cholesterol in the assembly of HRS-clathrin structures that suggests an existence of a feedback between the cholesterol-mediated HRS recruitment and HRS-mediated cholesterol immobilization; and (5) membrane-flattening ability of the GUV-associated HRS-clathrin assemblies in conditions of reduced membrane tension.

We thank this reviewer for the overall positive assessment of our manuscript, and we hope to have answered all her/his concerns in the following.

Besides these positive aspects of the study, there are also major problems. First, there is a serious mismatch between some data and the way the authors describe and interpret them, as explained in detail below. As a result, the major conclusions of this study are poorly supported. Second, the authors make little effort to mechanistically interrogate their findings. For example, some mechanistic insights into peculiar HRS behavior might be obtained by testing roles of individual HRS domains.

We have now tested the role of each HRS domain and region in formation of condensates by expressing GFP-tagged HRS constructs in HeLa cells. These data, which are now presented in Fig 2d-e, show that unstructured regions and the helical domain of HRS are important for condensates to form in cells. We discuss these new results in the main text (lines 183-211).

The specific concerns are presented below.

1. One main conclusion of the study is that HRS undergoes phase separation and forms liquid condensates. If it was so, these "condensates" would undergo fast turnover in the FRAP assay, but they poorly recovered.

We do agree that the poor recovery by FRAP seems incompatible with the liquid nature of the HRS condensates. This is a question of time scale. After carefully analyzing our data, we conclude that HRS forms gel-like protein condensates instead of classical liquid condensates. Gel-like protein condensates have a slow or negligible recovery in FRAP assays, as well as a slow fusion and dispersion rate (Lyu et al. 2021, PMID: 34508658). The fusion (Fig 1d) and spreading time on SLBs (Fig 1f) as well as slow recovery in FRAP assay (Fig 1e) are thus compatible with a gel-like behaviour of HRS condensates. Along the new figures 1d-f, we have edited the text to describe the gel-like behaviour of HRS condensates (lines 118-123, 129-131).

Also, phase separation indicates homogeneous-to-clustered redistribution, whereas HRS behaves in an opposite way: HRS clusters spread out into a more homogenous layer. However, there is a conflict here between the data obtained fluorescence microscopy (Figure 2A and video 3), where HRS spreads out, and the HS-AFM data (Video 4), where HRS clusters just appear from nowhere as if falling down from above. This discrepancy needs to be explained. Thus, the presented data show that rather than forming liquid condensates, HRS instead forms solid aggregates, probably, through some kind of strong self-association.

We observe that HRS condensates spread onto the membrane surface, but before they form a homogeneous layer, the condensates spread out from their contact point with the membrane and display a patchwork of HRS domains separated by areas of membrane with no HRS. Therefore, HRS remains phase-separated when spreading over the membrane, and the final homogeneous layer is formed by fusion/merging of the expanding HRS domains. These two characteristics (domains and fusions) are signatures of phase separation, showing that HRS when HRS is already phase-separated in solution, it wets the membrane in a phase-separated manner.

To further understand the phase separation behaviour of HRS in bulk and on membranes, we have performed experiments at lower concentrations. We now show that HRS can phase separate on membranes at bulk concentrations far below 1  $\mu$ M, in the range of 10 nM (see Figure 1h), while we found that it phase separates in bulk at concentrations in the range of 1-2  $\mu$ M. This fully supports the mechanism we propose, in which the very low saturation concentration onto the membrane leads to 2D phase separation only on the membrane. Furthermore, using our CRISPR-Cas9 cell line, we also have measured the cytoplasmic concentration of HRS-GFP cell line and found that it is 50 nM, showing that in the cell, HRS concentration is sufficient to phase separate on the membrane, but not in bulk.

Moreover, it explains the "discrepancy" between photonic and AFM data, in which a lower concentration has been used. In this case, the lower concentration explains the rapid apparition of domains when the density of HRS onto the membrane passes the threshold to phase separation.

We have added an example of 500 nM HRS recruitment on SLBs (Figure 1b). Moreover, to visualize the recruitment and condensate formation of HRS in low concentration on membranes, we have added a timelapse montage of 500 nM HRS recruitment on SLB imaged with AFM (Fig 2a). We have edited the main text to describe these data in more details (lines 140-163)

2. Another main conclusion in the study is that after binding to the HRS-coated lipid bilayers clathrin forms flat lattices. In fact, the most striking feature of the shown EM images, and the associated video, is that clathrin almost invariably forms small uniform spherical or hemi-spherical structures, which are tightly packed next to each other. Nowhere can one see extended flat clathrin lattices with a continuous hexagonal pattern, as it should be for flat clathrin lattices. It is true that the areas outside these collections of round clathrin cages have a rough appearance, which is likely explained by the presence of HRS. The authors need to show the structure of the HRS coat in the absence of clathrin. Even without this control, it can be inferred from the AFM images in Figure 2D that HRS by itself forms a rough surface, consistent with its ability to form aggregates. The small spherical clathrin structures shown by the authors strongly resemble the empty cages of ~80 nm in diameter, which clathrin forms in solution by itself. It seems that such cages are formed here, whereas HRS links them to the membrane and somehow to each other, often as a monolayer of cages. It would strengthen the study if the authors figured out how HRS does so, for example, using various HRS mutants.

We thank the reviewer for pointing these apparent discrepancies, making us realize that the presentation of our data may be misleading. We would like to take this opportunity to explain in detail the parts of the images that one needs to focus on in order to understand our point.

While it is difficult to know which exact figures this reviewer is referring to, she/he is right in saying that there are areas of PREM where a lot of spherical structures are visible. However, the area that are the most striking to us are the one coated with clathrin and that stay flat. On those structures, no hexagonal patterns are visible, even though the appearance of the protein layer is clearly rougher than the control of HRS alone (now shown in figure 4d and supplementary video 7). This indicates that on flat HRS condensates, clathrin does not polymerize into large hexagon/pentagon coats. This is supported by the AFM images as well, where only a few hexagons or pentagons are visible, scattered onto the flat surface (see Extended data fig 4).

The spherical cages are formed mostly onto droplets, and also onto upper layers of multi-layered bidimensional HRS condensates. They are not formed on the thinnest and first layer of HRS, nor at low densities of HRS where no condensate is forming. This shows that the droplets of HRS condensates that can be deformed allow the spherical cages can be formed, but that the flat and thin nature of the bidimensional HRS condensates impedes the formation of spherical cages. We exclude the possibility that cages are formed in solution as clathrin is added at a concentration where it does not spontaneously form cages, and that HRS is washed out of the chamber before the addition of clathrin.

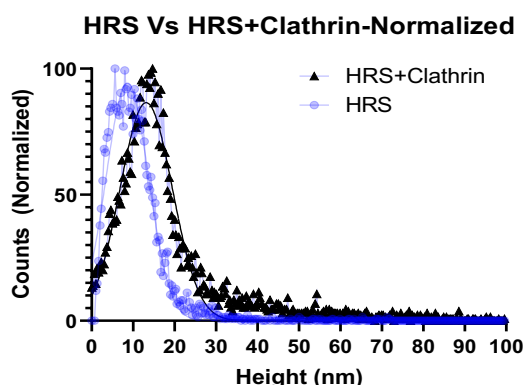
3. Yet another main conclusion of the manuscript is about the multilayering of the HRS-clathrin complexes. However, this point is unclear and rather ambiguous. From platinum replicas (Extended figure 2), it seems that by multilayering the authors mean that the flat layers of small clathrin cages sit on top of each other, in which case the thickness of a single layer should be similar to the cage diameter (~80 nm). However, this raises the question of whether HRS is present in the upper layers, as the authors propose multilayering of the HRS-clathrin complexes, but the provided images are inconclusive on this point.

We do agree that the multi-layering aspect of the HRS-clathrin structure remains unclear. We would like to precise the points that we know. First, we point out that multilayering is observed in the three high-resolution techniques we used, PREM and AFM for in vitro reconstitution, and CLEM cryo-electron tomography for in vivo. Importantly, the thickness of the layers is the same as measured by AFM in vitro and CLEM cryo-electron tomography in vivo, supporting the notion that assembly of clathrin and HRS is sufficient to create these multilayers and that the multilayers in vivo are similar to the ones in vitro. This thickness (roughly 50-60 nm) is, however way too small to be layers of spherical cages. Also, in vivo, we could only identify the bottom layer nearest the membrane as clathrin, in fact in a distance to the membrane expected for clathrin. In vitro, as pointed out by the reviewer, clathrin seems to cover the top part of all the layers. In vitro, HRS forms a two-dimensional condensate between the membrane and the first clathrin layer, but it is difficult to know if it is present in between subsequent clathrin layers.

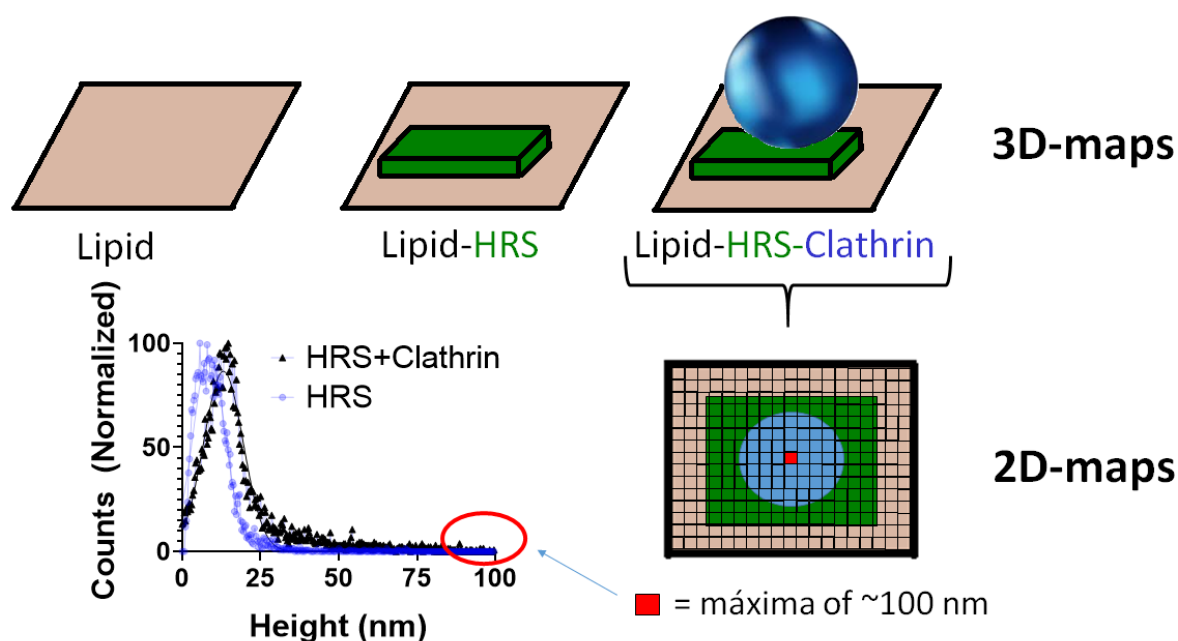
While we agree that more investigations should be made to fully understand the process of multilayering, we clearly show that the formation of these multilayers is strictly dependent on both clathrin and HRS, and that the appearance and dimensions of these in vitro multilayers match those in vivo.

More importantly, the authors find by AFM (Figure 3d,e and extended figure 5) that the thickness of a layer is ~10 nm. What is this layer then? In fact, the AFM images do not give an impression of layering, but rather of a very rough landscape. Also, the quantitative results showing distribution of sample heights poorly match the images. According to the colored scale bar in Figure 3D, the white peaks, which are quite numerous, should be ~100 nm high, but such numbers are not seen in the graph in Figure 3E. Also, the authors do not provide details of how the heights were measured. What kind of regions of interest were used? How were they selected? How many of them were quantified? How do the statistics on these measurements look like? Given that so many peaks and valleys are seen in the image, it is hard to imagine that the difference between 10 and 20 nm is significant.

We appreciate the reviewer's detailed analysis of the AFM data, which allows us to clarify our quantification and methodology. The 10nm thickness mentioned is specific to the HRS-only assembly, whereas the 20nm value, which is consistent with cryo-electron tomography data, corresponds to the full Clathrin + HRS assembly shown in Figure 3h-i and Extended Data Figure 4. We acknowledge that the raw topographs may appear as a "rough landscape," and we stress that layer thickness is measured as the height difference between the reference lipid membrane baseline (identified in flat areas and valleys) and the top of the assembled protein structures. Crucially, the tall peaks (above 100nm) were excluded from thickness quantification as they are not specifically associated with Clathrin or HRS but likely represent non-specific protein/lipid aggregates, which cannot be removed in AFM surface assays. To address the quantification details, we have updated Figure 3i to cover the full spectrum and have added explicit details to the figure legend and methods: height profiles were selected post-hoc from the membrane baseline to measure the layer height (thickness) across 2 HRS-only experiments and 5 Clathrin + HRS experiments, confirming that the resulting 10nm difference between the two assemblies is statistically significant.



The data appeared in histograms were obtained after applying a mask threshold and subtracting the lipid height contribution. Thus, all the AFM image pixels above the most external lipid surface were taken into account for HRS and HRS+clathrin conditions (not only the maxima of the feature but also the boundaries, please see the below-described schematic representation for the better illustration). For this reason, the relative percentage of height values near 100 nm are low in the condition HRS+clathrin.



**Schematic representation.** Workflow to analyze the height data distribution of Lipid-HRS and Lipid-HRS-clathrin conditions. The maxima height of clathrin cages is highlighted in red colour. It is possible to notice the low spectral density of the maximum of clathrin cages according to the rest of the feature boundaries.

4. It is not clear what exactly is similar between the reconstituted HRS-clathrin coats and endosomal platforms observed by cryoEM, as stated on p. 10, ll. 283-285. The authors found only one clathrin layer in the endosomal platforms in cells, as compared to what they claim as multilayering in vitro. The appearance and dimensions of

these two types of structures also look hardly comparable. The only similarity is that clathrin forms hexagonal lattices, which is hardly a surprise.

As explained above, the in vivo and in vitro platforms have in common to be made of HRS+clathrin, to be flat, to be multilayered, with a thickness of approximately 20 nm per layer. One apparent discrepancy is that clathrin appears quite disorganized (not hexagonal) in in vitro reconstituted assays, while we see a beautiful hexagonal pattern on the first layer of the in vivo platform. We however note that this hexagonal pattern, resulting from an averaging procedure, means that the predominant arrangement of clathrin is hexagonal, not excluding irregular patches of clathrin assembly. In any case, this pattern clearly signs the presence of clathrin in the HRS platform of endosomes. We address these points in lines 330-332:

*“However, we point out that because of the averaging techniques, the lattice may not be as regular as seen in the averages. A coat that is to a large extent unstructured but contains hexagonal elements, as seen by PREM, can result in a fully ordered, hexagonal average structure.”*

5. Quantification methods and statistical analyses for many sets of data are not provided.

We have modified Methods section and figure legends to address this comment.

Minor comments:

1. p. 6, ll. 176-177: “Notably, these protein patches did not show high-ordered structure, which indicates they form through phase separation”. There is no logic in this statement. A lack of ordered structure, like in any kind of mess, can occur without phase separation.

We have deleted this sentence.

2. p. 7, ll. 193-194: References cited here report true liquid-like condensates, which exhibit fast recovery and phase separation, and therefore they do not resemble the solid-like structures made by HRS, as stated by authors.

Indeed, our experiments with HRS condensates show that the protein forms viscous, gel-like condensates. We have edited the main text thoroughly to address this.

3. Figure 5F: Clathrin staining after npc1 KO needs to be shown. The authors also need to explain how they distinguished endosome-associated clathrin and plasma membrane-associated clathrin for quantification purposes, as well as provide other quantification details.

We have now added new examples of immunofluorescence images of HRS and clathrin in HeLa MZ and NPC1-KO cells (Fig 7c). We have also described more carefully, how the high-content analysis was performed in Methods (lines 932-936). Shortly, HRS-associated clathrin was analysed by thresholding HRS domains and using them as a mask to measure the HRS-associated clathrin intensities.

4. p. 13, ll. 404-408: The references on line 408 do not report the multilayering of clathrin on endosomes, as the authors claim in this sentence, except to some extent for the paper by Sachse et al. However, Sachse et al. report a “bilayered” structure of clathrin-containing platforms, but do not claim that both layers are made of clathrin. Instead, it is clear from the images that the two layers are different – the membrane-proximal dense layer is not a clathrin coat and might contain HRS, while the membrane distal layer is more transparent and resembles typical clathrin coats.

We agree with a reviewer that these references do not show multiple clathrin layers, but instead multiple protein layers on endosome membranes. With multilayering, we refer to this observation. Indeed, it is likely that HRS condensate forms the first, dense protein layer observed by Sachse et al. (also observed by Raposo et al., and van Niel et al., which we now cite as well) and clathrin assembles on the HRS layer, forming a second protein layer. Notably, the data by van Niel et al. shows similar coat dimensions to the measurements in our reconstituted samples (HRS layer 10-12 nm from membrane, clathrin layer 20 nm from membrane). In resin-embedded samples, the HRS layer is easier to distinguish because HRS accumulates the heavy metal stain. In our subtomogram averages HRS layer is not visible because it is not regular, but the distance of the clathrin layer is similar to that reported earlier (Van Niel et al.) and in our reconstitution samples. We agree that Raiborg et al. 2001, 2002 studies do not show a similar structure, highlighting the high complexity of these samples. We have edited the main text and references to highlight these points.

5. If authors intend to make their images accessible to color-blind readers, they should change the magenta+cyan combination to either cyan+red or magenta+green. Given that magenta is red+blue and cyan is green+blue, the readers still need to be able to distinguish red and green.

We have modified the figures based on this suggestion to make them more accessible.

6. Extended Figure 3a: Spell out the HRS domains and explain what they do.



We now introduce HRS domains in the introduction in lines 66-70.

7. Extended Figure 4b,c: Flip either panel B or C to align the line on B with the linescan in C.

We modified the figure based on this suggestion.

In summary, the authors present several intriguing observations obtained by technically powerful approaches but often provide misleading interpretations of their findings and, in most cases, do not achieve informative mechanistic insights into these phenomena. The information about quantitative approaches is largely missing. The study can be strengthened by an adequate description of the results and their proper discussion that should be consistent with the data, and by additional experiments that can provide at least some mechanistic insights into observed phenomena.

Reviewer #2 (Remarks to the Author):

This manuscript from the Roux and Kaksonen labs presents several pieces of very interesting experimental data which explore the ability of HRS proteins to form condensates, recruitment and assembly of clathrin at the surfaces of these condensates, the possible role of cholesterol and cargo in the process, and the possible implications of these observations for endosomal clathrin assembly and endosomal cargo sorting. My overall impression is that these are high quality and significant data that should be published in a visible venue such as Nat Comms. However, I feel the authors did themselves a disservice by presenting their data in a convoluted and confusing manner. I think the paper will appeal to a broad audience much better if it is reorganized and potentially shortened. Below I offer some suggestions to hopefully help the authors in this process and make the most of their findings.

We thank this reviewer for her/his overall positive assessment of our manuscript, and we hope that the answers to the questions and text edits we made in the following will clear all off all her/his remaining concerns.

1. Early in the paper the authors should explain clearly what they mean by the “unique bi-layered organization” of HRS-clathrin. The description of the multi-layer structures is not quite clear. Are there multiple layers of clathrin or of hrs or of both? The authors refer the reader to the videos but these too are confusing. It would be helpful if the authors could explain in greater detail how they are interpreting the images/video and perhaps quantify some aspect of them to show that a multi layer structures is indeed present. I finally understood it (I think) after taking a close look at extended data Fig. 2. The authors should describe this figure in detail in the main text as well as the quantification they performed there, none of which are mentioned at all in the text. The authors should consider moving this figure (or at least parts of it) to the main text to make this critical point clearer, earlier in the paper.

We thank the reviewer for this comment, which made us to realize that we indeed did not describe these structures clearly enough. Multilayering of the clathrin coat has, to our knowledge, not been observed before. Because we observe these structures only with above 500 nM HRS concentration, which is enough to promote the assembly of large, two-dimensional HRS condensates on membranes, we think that the multilayering arises from the property of HRS to form condensates. The mechanism of multilayering, however, remains ambiguous. It is possible that HRS forms multilayered condensates, on which clathrin then assembles. We however did not observe similar large multilayered structures with samples incubated only with HRS (without clathrin). Thus, the second option is that clathrin assembles on two-dimensional HRS condensates and through an unknown mechanism reorganizes the HRS coat to enable multilayering. As discussed in the main text, HRS contains two flexible, intrinsically disordered regions and a long helical domain between them. It is tempting to speculate that these flexible regions, which can expand to tens of nanometers away from “core protein” and also promote phase separation, are important for these multilayers to form. However, we acknowledge that future studies are needed to fully understand the mechanism of multilayering.

We have reorganized the figures and discussed the multilayering of HRS-clathrin coat in the main text to address this comment (lines 242-265).

2. Similarly, the description of the multi-layered protein structure on endosomes is interesting but rather confusing. The authors identify fairly convincingly that one of the layers is a clathrin lattice. What is in the other layers? Is it HRS? Are there other key proteins? It is not so clear to me that the results here are similar to their in vitro findings, where there were multiple layers of clathrin, perhaps interlaced with HRS. The authors need to more precisely justify their claim of agreement between in vitro and live cell results.

We do agree that the multi-layering aspect of the HRS-clathrin structure remains unclear. We would like here to precise the points that we know. First, we point out the multilayering is observed in the three high resolution techniques we used, PREM and AFM for in vitro reconstitution, and CLEM cryo-ET for in vivo. Convincingly, the thickness of the layers is the same as measured by AFM in vitro and CLEM tomography in vivo, supporting the notion that assembly of clathrin and HRS is sufficient to create these multi-layers and that the in vivo multi-layers in vivo are similar to the ones in vitro. This thickness of three protein layers combined (roughly 50 nm) is, however, way too small to be layers of spherical cages. Also, in vivo, the evidence that clathrin is present is only seen on the bottom layer, the one in contact with the membrane. In vitro, as pointed out by the reviewer, clathrin seems to cover the top part of all the layers, but it is difficult to know if it is present in between

the layers. As seen from the HRS alone control images, no multilayering is seen in absence of clathrin. This is fully supported by AFM data, where multilayers start to form when clathrin is added. Importantly, in images (Figure 4b) where the bottom layer is seen, the appearance of clathrin on this bottom layer is very different from the other layers. Clathrin is less organized and much flatter on the bottom layer, whereas it forms more spherical assemblies on the other layers.

While we agree that more investigations should be made to fully understand the process of multilayering, we clearly show that the formation of these multi-layers is strictly dependent on both clathrin and HRS, and that the appearance and dimensions of these *in vitro* multilayers match the ones *in vivo*.

3. Very importantly, the authors should explain the biochemistry of HRS interactions with clathrin at the beginning of the paper. In particular, what is the domain architecture of HRS and does it contain known binding motifs or domains for clathrin? I looked it up in the literature and found HRS has a well-established clathrin binding box. Astoundingly, this was never mentioned in the paper. Has HR been previously shown to assemble clathrin *in vitro*?

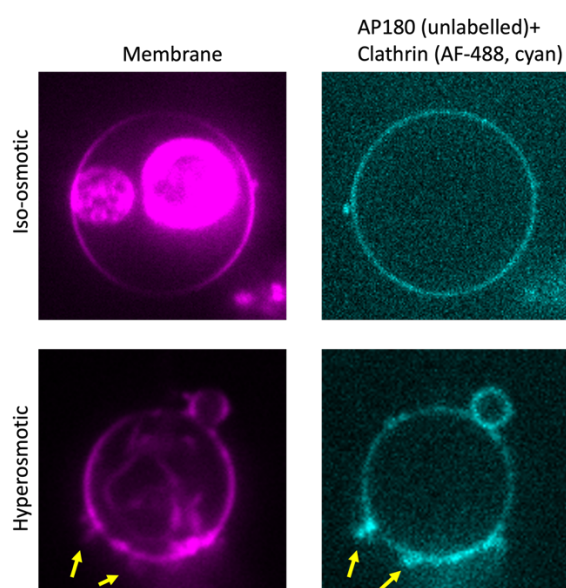
We have now introduced HRS and its domains more carefully in the introduction (lines 66-70). To our best knowledge, HRS has not previously been shown to assemble clathrin coat *in vitro*.

4. Also, what about the domain architecture of HRS might explain the authors' finding that HRS forms condensates? Not all disordered proteins form condensates. What is it about the amino acid composition of the IDRs perhaps that suggests phase separation? Have the authors examined the sequence using LLPS prediction software of which there are now many freely available?

We thank the reviewer for this comment. To better understand the mechanistic details how HRS forms condensates, we approached this question in two ways: 1) computational predictions, and 2) expressing truncated HRS proteins in cells. Our new data (Figure 2c-e, Extended data figure 3) show that HRS has two unstructured regions, with one of them having prion-like properties and enriched with glutamine residues. We designed GFP-tagged HRS constructs for mammalian cell expression to test which of these regions promote phase separation. Shortly, our new data show that the helical domain and the C-terminal prion-like region are important for condensate formation. Along new figures, we now discuss these results in the main text (lines 183-211).

5. The authors point out that their HRS-clathrin structures are flat in sharp contrast to earlier work with endocytic adaptors. It would be more convincing if at least one of these studies were repeated side by side with the current work to ensure that it is truly a difference in the proteins rather than a difference in the membrane preparation or the many other parameters that go into the preparation and imaging of these complicated samples. Some of the cited studies come from the same labs as the authors so it should be feasible. As ~10 years have passed and the lab members/materials/protocols are now likely changed, it seems a stretch to make a direct comparison with past data without repeating some part of it as a control.

We have now repeated this experiment with AP180 and clathrin (see the figure below), as described in Saleem et al. (PMID: 25695735). As expected, incubating GUVs containing 10% PI(4,5)P<sub>2</sub> and 15% cholesterol with 500 nM AP180 and 200 nM clathrin (20% labelled) did not promote membrane tubulation in iso-osmotic conditions. When we lowered the membrane tension of these GUVs with hypertonic shocks, we observed the outgrowth of clathrin-coated membrane tubes (yellow arrows in figures below). In these experiments, AP180 and clathrin did not promote similar flattening of membranes that we observed with HRS and clathrin under hyper-osmotic conditions. These experiments show that HRS-clathrin coat has unique properties that are not shared by AP180-clathrin coat.



6. To truly demonstrate that HRS can condense in 2 dimensions on the membrane, rather than falling down from solution, the authors should dramatically lower the protein concentration (10 to 100 fold) such that 3D protein condensates never form and then allow the membrane to become saturated in 2D with protein and spontaneously under phase separation, rather than simple wetting of the membrane by a 3D condensate. Previous work from several labs has shown that the saturation concentration on a 2D surface is typically 10-100X below that of 3D solution, owing to the reduced dimensionality. This is an important experiment, as the concentration of HRS in cells is likely too low for assembly of 3D condensates in the cytosol that can wet membranes.

We thank the reviewer for pointing this out. We have performed the experiments proposed by this reviewer and we now show that HRS can phase separate on membranes at bulk concentrations far below 1  $\mu$ M, in the range of 10 nM (see figure 1h), while we found that it phase separates in bulk at concentration in the range of 500nM to 1  $\mu$ M. This fully supports the mechanism proposed by this reviewer, in which the very low saturation concentration onto the membrane leads to 2D phase separation only on the membrane. Using our CrispR-Cas9 cell line, we also have measured the cytoplasmic concentration of HRS-GFP cell line and found that it is 50 nM, showing that in the cell, HRS concentration is sufficient to phase separate on the membrane, but not in bulk.

7. The authors state "Notably, these protein patches did not show high-ordered structure, which indicates they form through phase separation (Figure 2d)." This sentence should be amended or eliminated. The observation that a material lacks a high degree of order is not sufficient to prove how it formed through the process of phase separation.

We think our sentence was misleading, and we have now deleted it.

8. The sections of the paper on cholesterol feel tangential, as they are not well integrated into the rest of the story. It is not clear why there were included in this paper. I suggest saving them for a future study, as they seem to represent a separate topic that should be more fully explored on its own. The paper has plenty of data without these studies to warrant publication.

We think that the control of HRS phase separation by cholesterol is in line with this reviewer's suggestion that it is the protein density at the surface of the membrane that controls the 2D phase separation. Cholesterol seems to control how much HRS can be bound to the membrane, helping its phase separation. Therefore, it is an additional piece of evidence supporting the mechanism by which accumulation of HRS at the surface of the endosomal membrane, controlled by PI3P and cholesterol, triggers phase separation.

9. The abstract and introduction focus almost exclusively on flat clathrin lattices, while the authors' images show mainly curved lattices. I do not understand why the authors prefer to focus their interpretations on the flat lattices, which appear overall less apparent in the images.

Clathrin has a intrinsic propensity to form cup and cage structures, both in solution and when bound on membranes. What is interesting in HRS-clathrin coat is that it does not promote membrane bending, even with decreased membrane tension, as we show in Figure 8g-h. This is different from what happens with other clathrin adaptors, for example with AP180 and epsins (see above in reply to comment 5, and Saleem et al. PMID: 25695735). These observations indicate that, unlike in endocytosis where clathrin coat bends the membrane, HRS-clathrin coat is predominantly flat. In addition, our cryo-ET and subtomogram averaging experiments show that HRS-clathrin coat on endosomes in cells is predominantly flat.

10. I could not make much sense out of the final cartoon in Figure 7d. Why is the membrane budding? This is never clearly explained. What role is clathrin supposed to have in budding in the opposite direction of clathrin's curvature preference? This was never mentioned. I am sorry to be obtuse, but I got totally lost in trying to interpret this cartoon. A much clearer summary that is better grounded in the direct observations of the authors is needed.

We acknowledge that our model figure was too ambitious, and we have now simplified it. We think that the new model figure (and a schematic illustration in Figure 5c) now better summarize the main findings of the paper which are: 1) HRS forms two-dimensional condensates on membranes, 2) clathrin binds to HRS condensates, 3) HRS and clathrin form multilayered coats, 4) cholesterol promotes HRS recruitment on membranes, 5) HRS clusters cholesterol and cargo proteins under HRS-clathrin coats.

Minor points:

1. The arrangement of the first section of the paper is confusing. The authors start out with a complex system of membranes, HRS and clathrin. Then they go back to a simpler system of just HRS and membranes, then back again to the full system. The membrane composition is also changed several times in ways that are not very clear. For example, the composition of the first experiment is listed but then it simply says that 1% pi3p was used, which is the same as at first, so it is not clear what was changed. This is hard for the reader to follow. It would be easier to start with the simplest experiment and work up to the more complex experiment.

We thank the reviewer for this comment. We have reorganized our figures based on this comment to increase the clarity.

2. Various features of HRS (fyve domain, disordered regions) are mentioned at various places throughout the manuscript. It would be helpful to present the domain architecture of HRS from N to C in one place near the beginning of the paper. Doing so would increase the clarity of the story.

We have added a schematic illustration of HRS domain structure as Figure 2c.

3. The statement “cholesterol promotes membrane bending during CME” is perhaps too strong, as most studies are either correlative experiments in cells or highly simplified in vitro studies. Perhaps say instead “cholesterol correlates with membrane bending during CME”

We have edited the introduction based on this suggestion.

4. The authors comment that clathrin recruitment to membranes did not saturate at high HRS concentrations. How do the authors rationalize this observation? What is its significance?

This might explain the multilayered assembly of the HRS-clathrin coat. I assume that with a high HRS concentration, there is an excess of HRS to clathrin. As discussed above (Reviewer 2, comment 2), in theory it is possible for HRS to elongate through the first clathrin lattice and assemble a second layer on top of the first one. Non-saturating clathrin assembly might be an effect of multilayering.

Reviewer #3 (Remarks to the Author):

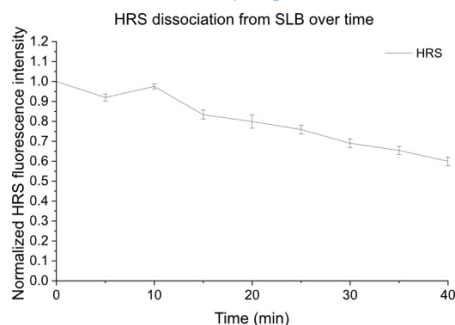
This work provides an interesting and convincing demonstration of a new mechanism for the formation of flat clathrin lattices through an elegant combination of reconstitution experiments and in situ imaging of endosomes by cryo-tomograph. The main findings are that the ESCRT-0 protein HRS forms condensates on membranes that promote clathrin assembly and that cholesterol plays an important regulatory role. This work provides new mechanistic insight in the function of ESCRT-0 in sorting ubiquitylated cargoes and it also provides an intriguing new example for a physiological role of protein condensates. The manuscript is well-written and the conclusions are largely supported by the data. The authors should address a few points for completeness and clarity.

1) In the HRS condensate experiments the authors use labeled HRS; they should show evidence regarding the question whether or not the label influences the HRS phase behavior.

We have now performed experiments with GFP-tagged HRS proteins in living HeLa cells. These data show that HRS forms biomolecular condensates also in physiological conditions in cells, and when tagged with GFP instead of chemical labeling. Further on, we note that when we performed experiments with labeled clathrin and labeled HRS, we mixed unlabeled protein with labeled protein in an 80:20 ratio, to minimize any unlikely effect of chemical labeling on protein behavior. We have edited the methods to address these points.

2) In their SLB assays, the authors sequentially incubate the SLBs with HRS and clathrin with an intermediate washing step. They should document and discuss the stability of HRS-membrane binding.

We tested the stability of HRS coat by washing out the bulk HRS protein after 30 minutes of assembly and then following the decay of HRS fluorescence over time. In the observation time of 40 minutes, the HRS fluorescence decreased around 30%. Because in our experiments, the time between the wash and clathrin addition is a few tens of seconds (up to 1 minute) and that clathrin assembly started immediately after addition, we do not think that HRS can significantly decrease and impact the results of our clathrin assembly experiments.



3) The description of the platinum replica EM data (Fig 1f) is very qualitative with descriptions of "frequent large patches", "often observed round clathrin islands" and "cup-shaped structures with various degrees of curvature". The authors should make these descriptions more precise and where possible quantitative.

We have now indicated the regions where clathrin coat forms mostly cup-shaped structures and those where clathrin coat is predominantly flat (now in figure 3g). We did not find an approach to measure the degree of coat curvature, and therefore the sentence "cup-shaped structures with various degrees of curvature" was deleted.

4) The authors should discuss in more detail the physiological relevance of their reconstitution assays. Specifically, they should motivate their choice of lipid composition and the protein concentrations and discuss the implications of combining human HRS and bovine clathrin.

Clathrin heavy chain is 100% identical between human and bovine clathrin. While post-translation modifications and light chains may be slightly different, we do not think this impacts greatly the results we have. We have chosen human HRS to be able to compare directly with experiments performed in human cell lines. In particular, as the critical concentrations to phase separate in solution or on membranes may depend on the precise sequence of the peptide, it was more important to be consistent in species with HRS than with clathrin. Based on our measurements, the cytoplasmic concentration of HRS is between 50-500 nM (supplementary figure 2). We have tested the recruitment of HRS to membranes as well as the recruitment of clathrin with similar HRS concentrations.

While the exact lipid composition of endosomes is not completely understood, we aimed to mimic the lipid composition of these membrane compartments in our experiments. Endosomes are enriched with PI(3)P phosphoinositides. We used 1% PI(3)P in all our experiments.

These points have been added to lines 101-103, 156-163, and 215-217.

5) The viscosity of the HRS films should be more carefully interpreted. The authors show FRAP data that suggest that recovery is slow. Did they check that the SLBs themselves are fluid? Does the fluidity of the HRS film change over time? Can they estimate a lower bound for the fluidity and is this consistent with the time scale (and ability) of the HRS droplets to fuse? Is the fluidity of membrane-wetting HRS films the same as the fluidity of bulk HRS droplets? Is the HRS film in fact fluid or a gel? In the discussion, the authors briefly speculate that the HRS-clathrin coat may be flat due to high rigidity of the HRS condensate. This should clarify this point and if possible investigate the mechanical properties of the film in more detail.

We thank the reviewer to give us a chance to explain this. We do agree that the poor recovery by FRAP seems "a priori" incompatible with the liquid nature of the HRS condensates. This is a question of time scale. The spherical shape, the fusion/fission behavior as well as the spreading onto the membrane do show they are liquid. But they are highly viscous, as these happen on time scales of tens of minutes. The FRAP is testing the viscosity at time scales of a few tens of seconds, where the condensate appear to be solid. These findings are thus compatible with a gel-like behaviour of HRS condensates, liquid at long time scales and solid at short time scales. We have modified the text to address these points (lines 116-123).

6) In measurements of cholesterol diffusion under HRS and HRS-clathrin coats, the authors report that cholesterol diffusion is more reduced under HRS-clathrin than under HRS alone. They should discuss why they think this is the case.

We thank the reviewer for this comment. While this is an interesting observation, we can only speculate about the possible reasons. One option is that clathrin reorganizes the HRS coat upon interacting with it, which leads to increased stability of the membrane underneath. It is possible that clathrin increased the gel-like properties of HRS, which would lead to increased phase separation of cholesterol and other lipids. This could explain why the cholesterol is found to be less diffusive when clathrin is added. Future studies on how HRS decreases cholesterol diffusion will hopefully give more complete answer to this question. We have now discussed these points in lines 428-430.

7) For the data on cargo cluster recruitment to GUVs (Fig 7) the authors should supply quantitative analysis of their claims that cargo clusters appear larger and more stable in presence of cholesterol.

While analysis of cargo cluster mobility is technically challenging, we have addressed this question by producing kymographs of HRS-cargo cluster movement on membranes. These kymographs show that the HRS-cargo cluster is large and immobile with membranes containing 30% cholesterol, while with membranes lacking cholesterol, these clusters are small and have increased mobility.

8) the authors should clarify their statement on p18 line 564 "To avoid unwanted assembly of clathrin cages in solution, all experiments were performed at physiological pH and salt concentrations". Furthermore, they should specify the "microfluidic device" that they mention.

Clathrin is known to self-assemble into polyhedral lattices in low pH (Brodsky 1988, Science), and in physiological pH the assembly happens only in the presence of clathrin adaptor proteins. We have modified the main text (Methods) to address these comments (lines 641-643).



9) in Fig 1B: why is the signal in the membrane channel grainy and why does this graininess increase over time (and which lipid is labeled)? And in Fig 1D: how is the nucleation rate determined from the data in Fig 1C?

This is a merge image with membrane in magenta and clathrin in cyan. The granular aspect comes from the clathrin signal, membrane signal remains constant over time. We have edited figures and figure legends (now Fig 3b) to make these points clearer.

We have also updated methods to explain how nucleation rates were calculated.

10) In Fig 2D: why is there a membrane island on the mica and what is the significance of this? AND in Fig 2E: what is the meaning of "High area"? (similar in Extended Data fig 5: what are "high region and low coat regions"?)

Large unilamellar vesicles (LUVs) were formed with the formulation detailed in the respective Materials & Methods section. Then, the vesicles explode when they adhere onto a flat solid surface under hypotonic buffer conditions, forming supported lipid bilayers (SLBs). This process is promoted if the substrate surface is negatively charged as freshly cleaved mica surfaces. The lipid concentration was optimized to 1 mg/mL to form SLBs that partially cover the mica surface, leaving regions of exposed mica. This partial coverage is critical to enable visualization of clathrin lattices on HRS condensates, as complete lipid monolayer coverage would obscure these structures.

The "high area" and "low area" regions correspond to clathrin cages observed at distinct height levels on the mica surface. These height differences reflect the assembly of the clathrin coat on HRS condensates, supporting the hypothesis of heterogeneous HRS-clathrin coat populations.

11) Fig 3e: is this histogram for the image in Fig 3d? And how representative is fig 3d-e?

The histogram in Fig. 3d correspond to the AFM imaging data shown in Fig. 3e (now Fig 3h-i). The topography changes after clathrin injection, and is representative of all scanned sample regions. As answered for other Reviewer concern the population size was enlarged (N = 2 and N = 5 for HRS and HRS+Clathrin, respectively) and similar results were found in all scanned samples at the same conditions.

12) Fig 6c/D: why is the scaling of the intensities with cholesterol mol% different for HRS and clathrin?

The labelling efficiency of HRS and clathrin are different, and thus the intensity values between HRS and clathrin are not comparable.

13) Fig 7B: the authors should annotate these images and explain more clearly what can be concluded from these data

We have now explained the data in the main text in more details.

#### Reviewer #4 (Remarks to the Author):

In my opinion this manuscript is well-written, technically sound and contributes to the field of cellular and structural biology by providing new insight into mechanisms of vesicle budding driving by ESCRT. I admit I am not an expert in Membrane re-modeling or ESCRT but to me, it seems fine to publishing this manuscript in Nature Communications. However, I have one major concern regarding the cryoET analysis and a few minor concerns with the figures. With regard to the cryoET, I feel like the analysis is lacking can should be addressed in the revised manuscript. Some of my points are minor and might be best addressed in the letter to the editor and reviewers.

We thank this reviewer for the positive assessment of our manuscript. We hope to have answered all her/his concerns in the following.

#### Main Suggestions for improving the manuscript

##### Major points:

1. In the abstract "Correlative cryo-tomography of HRS-labeled endosomes revealed a pure hexagonal lattice, consistent with flat clathrin structures."

It's not obvious to me that the cryoET data shows this. When I read the abstract, I was expecting to see multiple hexagons in the tomograms but I did not.

We have rephrased this sentence in the abstract to read:

*“Correlative cryo-electron tomography of HRS-labelled endosomes in cells revealed a multilayered structure containing a flat clathrin layer 16 nm away from the membrane, consistent with our in vitro findings.”*

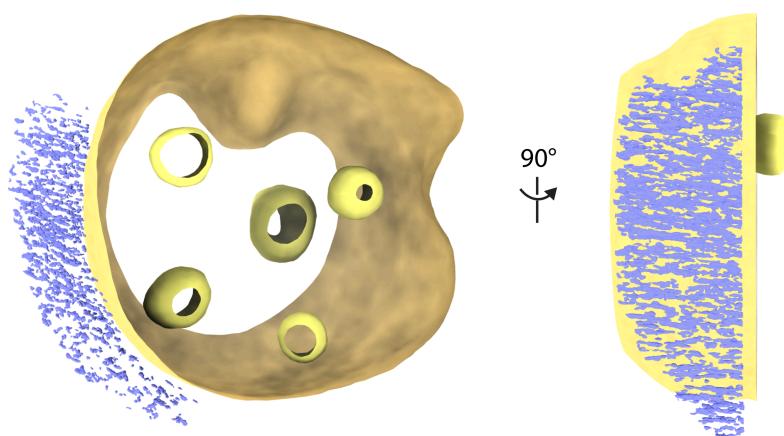
We realised that “pure hexagonal lattice” was misleading. We did not mean to imply that the clathrin lattice on the endosomes is entirely hexagonal, but that the fact that we reveal hexagonal arrangements allowed us to identify this particular layer as clathrin.

The lattice contains many hexagonal elements, resulting in a hexagonal appearance of the subtomogram average. There may be irregular clathrin arrangements also contained in the clathrin patches. Regular pentagonal or other geometries might also be contained but are likely minor populations as the hexagonal arrangement dominates the average.

Would it be possible to (1) segment the tomograms to show the different proteins and membranes, (2) use Isonet to denoise and fill in missing wedge information for the extracted subvolumes, and (3) superimpose the subvolume average into a tomogram using the XYZ coordinates of the subvolumes that were used to generate the final map (ie the IMOD command clone model). I think the segmentation and use of Isonet (or something similar) are common practices for cryoET aimed at characterizing unstructured cellular components.

The Reviewer asks about visualisation of the coated membrane by various means. In fact, subtomogram averaging achieves this in the most unbiased and quantitative way. Subtomogram averaging combines information from a set of tomograms, which is more powerful than analysing individual tomograms by segmentation or denoising.

(1) Segmentation depends on direct visibility and works particularly well for membranes. For a protein assembly such as the three-layered coat on endosomes, the assignment of density is arbitrary and therefore not unbiased. It is not possible to unambiguously assign the different proteins or layers within a segmentation model. See figure below; while the segmentation gives a sense of three-dimensionality and reveals membrane morphology, it does not convey more information on the coat than the tomographic slices in main Figure 6.



(2): Isonet has been successfully used to restore membrane continuity, for example of vesicles affected by the missing wedge. It is not generally established for filling unknown missing wedge information, and it is not advisable to apply Isonet before extracting subvolumes.

Importantly, subtomogram averaging fills missing wedge information by aligning and averaging subvolumes that have various orientations relative to the tilt geometry. Therefore, we were able to distinguish the three layers and to identify the clathrin layer using subtomogram averaging (see also point 2 below). Importantly, the hexagonal pattern appeared without applying any symmetry (see also Minor point 6)

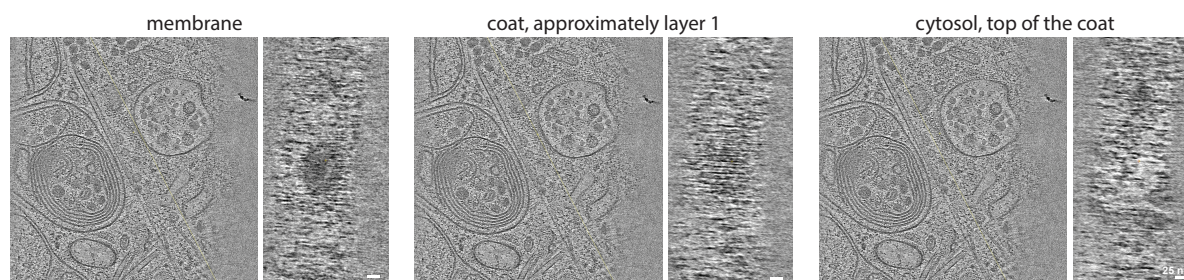
(3) Placing average maps back into tomograms is very informative and works well for discrete particles. However, given that the clathrin layer is a continuous, non-discrete assembly, the average is a representation of a patch of lattice, rather than an individual repeating unit. Superimposing it to an individual specific tomogram could be misleading and imply a “pure hexagonal lattice”, which we now want to avoid in the revision.

2. I don't see any signs of the hexagon lattice in the tomographic data only the sub-volume averaging. Do you see this in the tomographic data? If you see ordering can you modify the figures to show this? If you don't can you explain why? I think that might be due to the orientation of the endosome membrane relative to the XYZ of the tomographic reconstruction. Can you confirm? What does it look like when you rotate the tomogram so we see the XZ or YZ tomographic slice? Or maybe show a selected area FFT in the tomographic slice to show ordering. I think this is an important point to consider for this paper given that the main conclusions are based on characterizing the structure organization of the ESCRT-0 in vivo and situ.

This is a fair point. We do think that in large parts, the clathrin in the coat might not be structured, meaning that significant fractions of it do not form large hexagonal lattices in endosomes, but rather assembled into an unstructured layer. This is consistent with the fact from the 1809 subvolumes we extracted, after subtomogram classification, only 440 resulted in a class

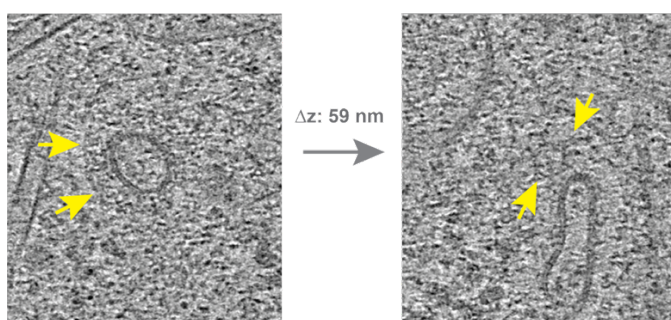
with a lattice-like appearance (without application of symmetry). This means that possibly only about 25% of the extracted coat patches are regularly organized. Furthermore, this is compatible with the fact that we do not see extended clathrin lattices directly in the raw tomographic data (see below). However, the averaging procedure allows to align and highlight hexagonal patches contained in the tomographic data. This piece of data is essential, because the lattice shows the presence of clathrin that cannot be evidenced by immuno-staining or labelling. But we do agree that the nice hexagonal lattice observed in the average data does not represent the structure of a single HRS-clathrin domain. We have made these points clearer in lines 888-904.

An additional difficulty for discerning the lattice in individual tomograms is the orientation of the endosome membrane relative to the xy-plane of the tomogram. In the perpendicular orientation, the anisotropic resolution of tomograms limits visibility. Thus, when the visualisation plane is rotated perpendicular to the xy-plane, the hexagonal pattern of the lattice is also not visible, but the coat density can be seen; see figure below which shows three different slices perpendicular to the xy-plane of the tomogram, positioned along the yellow lines in the left image panels. The central panel corresponds approximately to the position of the clathrin layer.



The main reason limiting visibility perpendicular to the xy-plane is the missing wedge of information. Furthermore, cryo-ET data has an inherently low signal-to-noise ratio caused by the limited exposure to the electron beam. In cellular cryo-ET, low contrast and noisy appearance are additionally caused by the macromolecular crowdedness of the cytosol. Therefore, in many cellular regions, the dense packing of diverse macromolecules makes it difficult to distinguish individual structures, even if these structures are ordered. Conversely, when the surrounding is devoid of other macromolecules (i.e. diluted), individual structures become visible as they seemingly have a high contrast. This also affects clathrin lattices. Although hexagonal lattices can be directly observed by cryo-ET in some cases, there are many cases where the lattice is difficult to see as a result of the dense surrounding. An example is shown below – a typical clathrin coated bud on an intracellular membrane, in one of the tomograms of our data set used in this manuscript. The left image shows a side view of the clathrin coated bud, the right image a different virtual slice showing the surface of the bud, revealing a few hexagonal elements. Despite these hexagons being positioned in a favourable orientation relative to the tomogram geometry (i.e. in the xy-plane), they are difficult to discern. Note that for the HRS-clathrin structures, the hexagons are directly “covered” by another protein layer, probably resulting in a higher local macromolecular density than for the clathrin coated bud seen here.

**All these effects are mitigated by subtomogram averaging**, which allows to fill the missing wedge information, increase the signal-to-noise ratio, and “average-out” the non-regularly arranged macromolecules obscuring the clathrin lattice. Integrating data from multiple tomograms by subtomogram averaging therefore allows to reveal otherwise elusive structural features.



For a selected area FFT / power spectrum to show a hexagonal lattice distinguishable from the background, a substantial number of ordered repeating units have to be present in the selected area. However, the patches of coat we are dealing with are relatively small, about 100- 200 nm in diameter, thus containing probably less than approximately 30 hexagons. Therefore again, it is only through subtomogram averaging that the lattice becomes distinguishable in a power spectrum.

And clarify in the manuscript what you mean by a pure hexagonal lattice.

Please see response to point 1 above. In addition to the abstract, we have clarified the main text. In the Results, we edited the following text passage:

*The averaging indicated that the predominant feature of the first layer was a hexagonal lattice. We therefore applied a six-fold symmetry, which revealed a lattice constant (vertex-to-vertex length) of 24-26 nm (Figures 6f and Extended Data Fig. 7e). These dimensions and the hexagonal organization are typical for flat clathrin lattices (Sochacki et al., 2021; Fotin et al., 2004; Kravčenko et al., 2024), indicating that this layer corresponds to a flat clathrin coat. However, we point out that*



*because of the averaging techniques, the lattice may not be as regular as seen in the averages. A coat that is to a large extent unstructured but contains hexagonal elements, as seen by PREM, can result in a fully ordered, hexagonal average structure. The same analysis of the second and the third layers did not reveal periodical arrangements that would be indicative of a lattice-like structure.*

In the Discussion, we added the sentence:

*Other arrangements, such as pentagonal elements, might be contained in the lattice but are likely less abundant and thus not represented by the average.*

Minor points:

1. Figure 1. What happens if you add HRS to the membrane and image using PREM? It would be helpful for me to understand why this was not done. From reading the manuscript it seems like the authors could have done this experiment to gain a better understanding of how HSR organizes on a PM in the absence of clathrin. Maybe they have done this experiment in a previous publication and it's not necessary for this manuscript or it is common knowledge in the ESCRT field that I am not aware of. If that's the case can the authors address this point in the letter to the editor and reviewers?

We have now added the PREM images of 1  $\mu$ M HRS on SLBs as new Figure 4d. The appearance of HRS alone coat differs from that of HRS+clathrin coat. HRS coat does not show any clear organization, which might explain why it was not resolved by subtomogram averaging.

2. Figure 1. I can see the clathrin cages in the PREM images but the flat clathrin patches seem disordered and do not have any signs of symmetry. Can the authors confirm? It's not obvious how closely the PREM images of the flat clathrin patches resemble the subvolume average about symmetry.

We agree with this reviewer that flat clathrin coats are less organized than curved ones. However, the height of our reconstituted HRS-clathrin coats are very similar to those we observed on endosomes in HeLa cells. Moreover, we could not distinguish clear hexagonal pattern in our CryoET images, but the coat structure was revealed only after subtomogram averaging. We therefore think that our reconstitutions replicate the behaviour of HRS-clathrin coat in cells. We however acknowledge that further studies are needed to explore the structure of HRS-clathrin coat, including the origin of two currently unknown proteins layers on endosome membranes.

3. Figure 4. Is it possible to show an image or pseudo color or trace around the PREM images to indicate where the (1) low-density HRS, (2) two-dimensional HRS condensates, and (3) droplet-like HRS condensates are? The cartoon is helpful but just from looking at the PREM images, it is difficult for me to see where the boundaries of each are.

While distinction between clathrin populations between HRS droplet condensates and two-dimensional condensates is rather clear, it is less easy to see in our samples where the border between clathrin coats assembled on two-dimensional condensates and low-density HRS are. This is because the bulk HRS concentration to promote droplet condensate formation has to be high, and high HRS bulk concentration leads to high HRS recruitment on membranes also outside the condensates. We therefore prepare low-density HRS and HRS condensate samples separately.

4. Figure 5. I don't see any hexagonal clathrin structures in the high-mag AFM images in Fig 5c-d. Can you confirm if there are any hexagons? And can you add an arrow over (maybe just one or two) the hexagons and the pentamers? I think the high mag insets should be larger so that it is easier for the reader to see the hexamer and pentamer. Even when looking at the pdf on my 27-inch computer monitor I have to zoom in and squint my eyes to see the pentamer and I don't see any hexagons.

We have enlarged the figure to make it more readable. We agree that the resolution does not make it easy to identify hexagon and pentagons from the HS-AFM data. The main reason for this is based on the nature of the scanning mode used for image data acquisition. Fast-QI measurements were carried out to minimize the non-desirable frictional lateral forces that could not only drag the soft clathrin cages but also leading to tip contamination. This mode operates performing a single force-distance curve at each image pixel to extract to topology dimensions. The deformation underwent by the clathrin cages under external axial loads even applying low regime of forces. This fact is not surprising since the clathrin polygon architecture is compressed (Jin et al. 2006, PMID: 16473913). This observation underlines the high tendency of triskelia to form regular polygons under external load forces bending the triskelia arms to more energetically favorable pentamer conformation. As a result, the formation of nearly-closed spherical clathrin cages is subjected to substantial distortions of the triskelion arm boundaries by the load forces exerted during the QI data acquisition. While the imaging setup makes it difficult to distinguish pentagons and hexagons, we have indicated some structures we recognized as hexagons with blue arrows.

5. It's not clear to me how well the fLM and cryoET data correlate. Can you modify Extended Data Figure 7 to show an fLM image next to a tomographic slide corresponding to pan d ? It's difficult for me to tell which of the tomograms is associated with panels a-d and how well the fluorescent signal matches panels e-p concerning the yellow arrows

pointing to the putative protein coat on the endosomes. In one of the CLEM papers, the authors reference Ganeva et al., 2023 do a fine job of showing CLEM. In this paper, the authors superimpose the fLM image with a tomographic slice (figure 1) to show how well the images correlate.

Panel D of Extended Data Figure 7 (now Ext data Fig 6) was a part of the cryo-EM overview map of the lamella, and the yellow square indicated the area corresponding to the tomogram that is shown in the main Figure 4A (now Fig 6a). We agree with the Reviewer that this view can be confusing, especially since the contrast in the overview map is very low. **We have therefore replaced panel D with a virtual slice of the respective tomogram.** For this, we used a xy-slice from a different z-position than the slice shown in Figure 6A, but it is from the same tomogram.

We prefer not show overlays of cryo-FM and cryo-ET data, as the scales are very different and thus overlays are not very informative. Therefore, the correlation involves the overview maps as an intermediate to base the tomogram acquisition on. For the same reasons, we have also not shown such overlays in Ganeva et al 2023. However, there, the two-colour fluorescent signals reveal the characteristic shape of lipid droplets, making a visual correlation easier.

6. Extended Figure 8. Can you show an image of the subvolume average (440 subvolumes) with C1 symmetry? I appreciate the authors showing the initial subvolume with C1 but wonder how much the symmetry helps or how much of the appearance is due to imposing symmetry.

The average shown in Extended Data Figure 7A was in fact obtained from the final 440 subvolumes without imposing symmetry, while the average shown in Extended Data Figure 7B and in Figure 6F was obtained with C6 symmetry from the same 440 subvolumes and after refinement.

We have now clarified this in the legend to Supplementary Figure S7:

*(A) Side view (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 sideview images and 40 nm for top-view images. (B) Side view (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.*

7. Figure 7d seems out-of-place in figure 7, can you make it a new figure?

We have made a new figure that connects better with the cargo sorting assays presented in this figure.

8. Are you going to depositing the subvolume average to the EMDB?

Yes, we will deposit the subtomogram average as well as the tomogram shown in the Figure to the EMDB; the depositions will be accessible upon publication.

## REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The revised manuscript is significantly improved. The authors added new experiments with HRS deletion mutants and with low concentrations of HRS. They more adequately described gel-like properties of the HRS condensates, acknowledged the presence of clathrin cups and cages in the PREM images, as well as a lack of regular clathrin organization in the flat regions. They also showed the topology of the membrane coated with high concentrations of HRS (1  $\mu$ M for PREM and 500 nM for AFM), partially clarified the multilayering phenomenon, added statistics and addressed my previous minor comments.

We thank the reviewer for comments and suggestion that helped us to improve the manuscript.

Despite these improvements, I am still not convinced that AFM data are consistent with one of the main points of the manuscript that upon condensation HRS forms a 2D layer. In the rebuttal letter (although not in the manuscript), the authors explain the sudden appearance of HRS bumps in AFM images as events of fast HRS condensation when “the density of HRS onto the membrane passes the threshold to phase separation”. If so, then HRS forms 3D aggregates instead of 2D layers. How is it consistent with the 2D layer hypothesis and with the data that suggest that HRS condensates are viscous and thus are unlikely to form instantly?

The appearance of larger protein condensates in AFM data is in line with our fluorescence microscopy samples. In Supplementary video 2, spherical protein condensates formed in solution sediment on membranes. These spherical condensates then disperse when the 2D condensate spreads on the membrane. The fact that these droplets undergo dispersion shows that they are biomolecular condensates instead of aggregates. Based on our data, HRS can form 2D condensates in two ways: 1) by interacting directly with membranes, and when local concentration exceeds the phase-separation threshold, the protein forms 2D condensates directly on the membrane. 2) In high concentrations, HRS forms spherical condensates in solutions, which, when interacting with membranes, disperse. The dispersal of spherical condensate on the membrane leads to 2D condensate. The AFM experiments were performed in a 500  $\mu$ M HRS concentration, which favors the direct phase separation on membranes over phase separation in solution. We show that in AFM experiments, protein first binds to the membrane in a homogenous manner (Figure 2a, timepoint 40 min), and slowly forms protein islands (Figure 2a, timepoint 61 min). Notably, the timescale in AFM experiments is similar to that in fluorescence microscopy experiments (Figure 1g), minutes rather than seconds. This indicates that above a critical concentration on the membrane, HRS slowly forms viscous, two-dimensional, gel-like condensates.

The sudden disappearance of these 3D structures, as seen in video 3, also remains unexplained.

We thank Reviewer 1 for the comment and for pointing out this technical issue. What is observed in Supplementary video 3 is a parachuting effect: during Fast-AFM imaging, we adjust the force and feedback gain applied to the AFM tip (similar to increasing the intensity of a confocal laser). We start with a very low force, which results in lower resolution, due to minimal tip-sample interaction, and causes proteins to appear larger. Once the sample was

stable, we increased the gain and force to improve resolution, which is also observable on the surface outside the membrane.

We have updated the video legend to point this out.

In the newly added explanation of how the authors measured the heights of structures in AFM images, it is unclear what the “mask threshold” was and what it selected and rejected in these images.

We apologize for not being precise enough here. What we mean by mask threshold is that we first imaged membranes on mica without proteins. We then identified the regions of membranes from these images using the threshold plugin tool in Fiji, and subsequently analysed the protein coat thicknesses from these membrane regions. The height of the membrane was subtracted from the final protein coat thickness. We have edited method sections as follows:

“ROI areas of membranes on Mica were identified using the threshold plugin tool in Fiji. The height of membranes areas was then measured and set to 0 nm. The protein coat thicknesses were measured by using threshold-identified membrane areas. The membrane surface height was subtracted from the final protein coat thickness.”

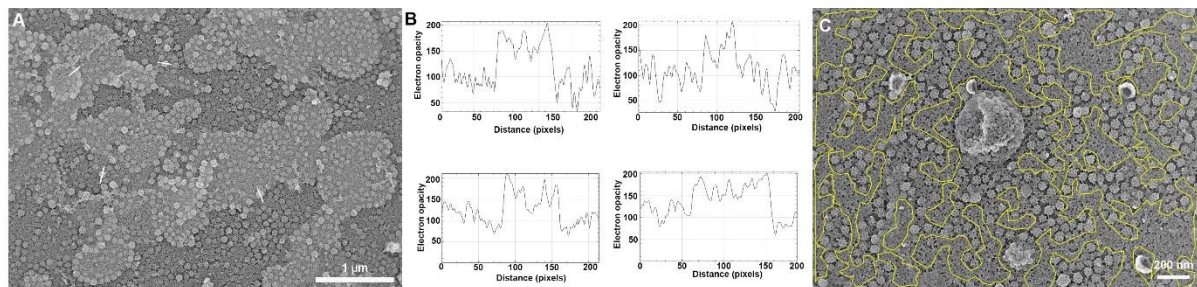
It is also hard for me to share the authors' enthusiasm about flat clathrin-containing areas, as compared with those coated by clathrin cups and cages. One of the authors' main conclusions is that at low HRS concentrations clathrin mostly forms flat layers (lines 441-444). They also illustrate this point by the diagrams in Figures 10F and 4C. However, the PREM images show that virtually every “flat” clathrin island contains a cup or a cage. Although the authors acknowledge this fact in the main text, they do not take it into account in their final conclusions. Consistent with PREM data, the AFM images (Extended figure 4C) also show clathrin polygons only on the “hill tops”, although authors describe them in the rebuttal letter as being “scattered onto the flat surface”.

Furthermore, since the “flat clathrin” areas, on which the authors focus their attention, are disorganized and do not show a clear polygonal pattern, it is inappropriate to refer to them as “coats” or “lattices”, as the authors do throughout the manuscript, and even conclude the manuscript with the statement “Clathrin is assembled as a hexagonal lattice on HRS, which forms two-dimensional condensates on membranes” (lines 522-523). Together, these data suggest to me that, at least in this in vitro reconstitution system, the flat clathrin arrays are very unstable and form cups and cages as soon as they have enough neighbours. Therefore, the authors need to somehow strengthen their conclusion on this point or change it. It could be worthwhile to quantify the fractions of flat versus cup-covered regions on 2D HRS layers either as a function of time or concentration of HRS or clathrin to get a sense which type of the array is more common.

First, we would like to point out that in our definition, coats are not necessarily structured, in contrast to lattices. We agree with the reviewer that the reconstituted clathrin coat is intrinsically unstable and prefers the cup-shaped structures instead of flat ones. This was also shown in previous studies (PMID: 12353027, PMID:11161218). To our knowledge, the flat clathrin coat was successfully reconstituted on the membrane only at +4°C temperature, and even then, the coat started to bend when the temperature was increased (PMID: 22522172). In our in vitro reconstitution experiments with HRS and clathrin, we observed both flat and dome-like protein coats. Therefore, we turned to cryo-electron tomography to visualize the HRS-clathrin coat in cells. These experiments revealed a flat coat on endosomes. Supporting this, the reconstituted HRS-clathrin coat did not bend the GUV

membrane even when the membrane tension was decreased (Figure 8g-h). These observations support our conclusion that, while in vitro HRS-clathrin coat can be either flat or dome-like, the natural conformation of the coat is flat.

Quantifying the curvature of clathrin coats from PREM images presents inherent challenges due to the existence of clathrin assemblies in various intermediate curvature states. To address this issue, we conducted an analysis comparing the relative abundance of flat versus curved clathrin regions, as depicted in Extended Data Fig. 4b, with the methodology detailed in the Materials and Methods section. A similar quantification strategy was employed in our previous study (Moulay et al., 2020), and comparable methods for distinguishing flat from curved pits have been documented (Grove et al., 2014).



Curved pits demonstrate higher apparent electron opacity compared to flat clathrin lattices, reflecting differences in electron beam transmission. In flat lattices, electrons traverse a single membrane layer, whereas in curved pits, the beam passes through multiple membrane layers, resulting in reduced transmission and increased opacity. Line intensity profiles across these structures exhibit a characteristic reverse W-shaped pattern (Fig. A, B). Based on this, regions were delineated and categorized as flat, and the pooled surface areas were quantified to calculate the percentage of flat clathrin structures relative to total clathrin structures (Fig. C).

It is important to note that this analysis does not quantify the precise degree of curvature but rather classifies clathrin coats into flat and curved populations. The results underscore the variability of reconstituted clathrin assemblies, and the main text has been revised accordingly to clarify this point.

The point of multilayering is still not explicitly clarified, especially when it concerns the comparison of layers in vitro and in vivo. The newly added PREM data showing the structure of HRS layer alone suggest that HRS at high concentration can form at least two layers in vitro suggesting the possibility that a single layer of clathrin can be formed on top of the HRS “sandwich”. In vivo, it seems that neither HRS nor clathrin form multiple layers, while additional layers seen in vivo are not relevant to what is observed in vitro. The clathrin arrangement is also different in these two conditions – flat hexagonal in vivo and unstructured flat or consisting of mostly pentagonal cups in vitro – the fact the authors also acknowledge. Thus, the only argument in favor of similarity between in vitro and in vivo layers is their similar thickness, which is not a strong argument. Another similarity – contribution of both HRS and clathrin – was already known before. Maybe I do not understand what authors mean by “multilayering”. Is one layer of HRS plus one layer of clathrin considered as multiple layers? If so, the terminology needs to be explained. On the other hand, how then to deal with this point in relation to the data shown in figure 4?



The multilayering of the protein coat on endosomes is highly unusual, and to our knowledge, this type of clathrin structure is observed only on ESCRT-0 microdomains on endosomes. First, we point out that the dimensions and the multilayered nature of the HRS coat are consistent between the in vivo and in vitro data. Also, as pointed out in our previous rebuttal, the apparent hexagonal lattice that emerges from averaging techniques from in vivo data may not represent the real structure of the coat, as unstructured parts of the coat may have been averaged out. As a matter of fact, in individual cryo-electron tomograms, we do not see hexagonal lattices, as we explained in our previous rebuttal.

Despite our efforts, we could not solve the two other protein layers, and more work is required to fully characterize these unusual structures. HRS condensates give one plausible explanation for the multilayering. Since the thickness of the reconstituted HRS layer matches that of the protein coat in cells, it is tempting to hypothesize that the HRS condensate expands beyond the first clathrin layer to form subsequent protein layers. Whether this is the case or if additional proteins are recruited to form additional protein layers remains to be studied in the future. To answer these questions, we will continue these studies in the future and will, for example, perform BioID interactomics experiments and super-resolution microscopy to map the proteome of these unusual protein coats. However, these experiments are beyond the scope of the current study.

We have emphasized the need for future studies to fully understand the architecture of multilayered protein coats on endosomes (lines 470-472 and 489).

Other comments:

1. Extended Data Fig. 2e needs a better legend or labeling. It is not clear what each panel shows.

We have modified the figure and figure legend based on this suggestion.

2. In Figure 2a, it is not clear which areas show “homogeneous binding of HRS” and which represent naked membrane. They should be labeled. What is the interpretation of the patches in these images?

We realized that the example montage image of the AFM experiment with HRS was not the clearest possible, because the borders of the SLB membrane and bare mica surface were difficult to see. We have now changed the montage to a clearer example and highlighted the borders of the membrane SLB with a red dashed line. As explained above, HRS first binds homogeneously on the membrane (timepoints 17min, 40 min, and 50 min, Figure 2a), and after reaching a threshold concentration, it starts to form dense condensates, which are visible as islands (timepoints 61 min and 73 min, Figure 2a). We interpret that these patches are similar to those protein patches observed with a fluorescence microscope (e.g. Fig 1a-b, Fig 1g-h).

3. The text (lines 171-172) says that Figure 2b shows “the thickness of the HRS patches”, whereas the legend to this figure says that it is “A histogram of HRS and HRS-clathrin coat heights”. The label just says it is membrane and “high area”. What is shown there?

We agree that using different terms here might be confusing. We now use the term “thickness” when we measure the distance of the protein coat surface from the membrane.

Similarly, we refer to “thick protein coat” and “thin protein coat” instead of “high region” and “low region”. We have edited the text and updated Figures 2b, 3i, and Extended data Fig 4, and respective figure legends. We have edited the graph in Fig 2b to make it clearer.

4. A control image of membrane coated with 100 nM HRS alone is still missing to accompany data in figure 3f. It is needed in order to properly interpret the image with HRS and clathrin.

We have added a control image of a sample with 100 nM HRS alone as a new Extended data Figure 4a.

5. Video 7 does not play.

We downloaded a new version of this video.

6. The text refers to “Figures 5 and Extended data Fig 5f-h, but the Extended data Fig 5 ends with panel f.

We have corrected this reference.

7. I do not understand the logic of the following sentence “without phase separation, the HRS-clathrin coat could grow indefinitely because it is flat.” With phase separation it is also flat. I also do not see why non-flat coat cannot grow indefinitely.

We have deleted this sentence from the Discussion.

8. Some Extended data figures remain in the cyan-magenta combination.

We have now modified these figures to be more colour-blind friendly

In summary, the authors present a large body of data, some of which are truly interesting and intriguing, but their main conclusions do not appear well supported by the data, and I still have hard time to formulate for myself what I have learned from this study. The data just do not come together too well to form a clear concept.

We hope that our answers to these comments have clarified our results and the importance of our findings. The multilayered protein coats on endosomes were first imaged over two decades ago, but their detailed organization and the mechanism of assembly have remained unknown. Here, we show that HRS forms two-dimensional protein condensates on membranes and that these condensates directly recruit clathrin. To our best knowledge, this is the first time such a direct interaction of clathrin with biomolecular condensates is shown. We accept that there are open questions remaining, most notably the nature of the other two protein layers on top of the clathrin coat in cells. However, our study enlightens many previously unknown aspects of this uncanonical clathrin structure.

Reviewer #2 (Remarks to the Author):

The authors have been very thorough and thoughtful in their response to my questions as well as those of the other referees. I feel the paper is ready to be published. Congratulations to the authors on a fine work.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily addressed the reviewer's concerns leading to a greatly clarified manuscript.

Reviewer #4 (Remarks to the Author):

All my comments and suggestions were addressed in the revised manuscript. The revised manuscript looks good for publication.

We thank all reviewers for critical comments and contribution that have helped us to improve our manuscript.



## REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I am generally satisfied with this revision. However, I suggest some editorial changes in order to improve clarity, correct typos and revise some statements that apparently have been inherited from the earlier versions of the manuscript and not edited in view of newer data.

1) p. 6, ll.131-132: “We noticed the spontaneous formation of HRS domains with the same intensity as the two dimensional condensate spreading out from droplets (Figure 1f, yellow arrowheads, Figure 1g).”

Please, clarify, what condition is being compared here to the spreading droplets. Although it can be deduced from the Figure 1g legend and subsequent text, it would be clearer to state it directly.

We clarified the main text by adding a sentence “When we incubated SLBs containing 1% PI(3)P (Lipid mix 1, Supplementary Table 2) with 2  $\mu$ M HRS, we noticed...”

2) p. 7, l. 161-163: “...readily spread on the DOPS/PI(3)P-rich membrane as well as membranes containing 20% DOPS but no PI(3)P (Lipid mix 2, Supplementary Table 2), forming a dense protein film (Extended Data Fig. 1e)”

It should be mentioned here that an additional difference for the middle and right panels in Ext. Fig. 1e is the presence of cholesterol, which is not present in the left panel. In the light of the data shown later in the manuscript on the role of cholesterol, the interpretation of these data could be not so straightforward.

We now mention in the main text that the two tested lipid mixes contain also cholesterol.

3) p. 7, l. 169: “...(Figure 2a, timepoint 39 min), which slowly separated into patches (Figure 2a, timepoint 64 min).

The figure shows timepoints 40 min, 50 min, 61 min and 73 min, but not 39 min and 64 min.

We have corrected the main text to refer to correct time point (61 min).

4) p. 10, ll 286-287: “Finally, we observed multilayered clathrin coats on two-

dimensional HRS condensates”

The authors rather showed single-layer clathrin coats (Figure 5 and Extended figure 6a-c) on multilayered 2D HRS condensates (Figure 4d).

As stated in the main text, “the electron opacity increased gradually, doubling in each new layer, indicating that these layers contain multiple layers of clathrin (Figure 4c)”. While the exact nature of these multilayered coats remains to be studied, our data indicates that clathrin can form multilayered coat on HRS condensates. We edited the statement as follows: “Finally, we observed multilayered clathrin coats on HRS condensates”.

5) p. 22, ll. 652-653: “Before and in between each protein addition, m”

This sentence seems truncated.

We apologize for this mistake. We corrected the sentence: “In between each protein addition, samples were washed five times with the reaction buffer.”

6) p. 47, l. 1383: “Scale bars in panel h...”

It should be panel a.

This was corrected.

7) p. 48, l. 1396: “Representative PREM images clathrin coat reconstituted...”

Should, probably be “...images OF clathrin coat”

We corrected this figure legend.

8) p. 48, ll. 1402-1403: “Two-dimensional HRS condensates lead to a predominantly flat clathrin coat.”

Suggested revision: “Two-dimensional HRS condensates lead to a mixture of flat and dome-like clathrin coats.” This is because the quantification newly included into the revision shows that dome-like coats represent ~80% of surface area and flat ones ~20% (Ext. Fig. 4b)

We agree with the reviewer that the suggested phrasing matches better with the data.

9) p. 50, l. 1462: “Sorting coefficient values were blotted...”

plotted?

This was corrected.

10) p. 50, ll. 1488-1489: “Clathrin assembles on HRS condensate to form a multilayered protein coat, where clathrin coat is predominantly flat.”

According to authors' data, clathrin forms a single layer (Figure 5 and Extended figure 6a-c) and is predominantly curved (80%, Ext. Fig. 4b). This statement need to be revised accordingly.

We edited the statement as follows: “leading to a mixture of flat and curved clathrin coats.”

11) p. 52, ll. 1541-1542: “Blue arrows indicate recognized hexagons.”

These arrows are in panel c not panel a.

This was corrected.

12) p. 52, ll. 1545-1546: “The scattered box indicates the region where the zoom-in image.”

Revise the sentence.

We corrected this figure legend.

13) p. 54, l. 1613: “...the gain and force applied to AFM tip for increased...”

‘was’ increased?

We corrected this supplementary video legend.