nature portfolio

Peer Review File

Lipid turnover and GEF recruitment collectively determine Rab5 recruitment and activation during the first step of early endosome formation

Corresponding Author: Dr Kangmin He

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.
Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this study, the authors address the mechanisms by which the small GTPase Rab5 is targeted to endocytosing membranes resulting in the formation of early endosomes. To this aim, the authors use sets of genetically engineered cell lines expressing tagged variants of Rab5 isoforms, various GTP-exchange factors of Rab5 and additional proteins involved in the formation and uncoating of clathrin-coated vesicles to not only arrive at a precise sequence of recruiting but also to pinpoint the recruiting mechanisms. Using advanced and automated time-lapse imaging, the authors not only show that the two GEFs Rabex and hRM6 cooperate to recruit Rab5 immediately after uncoating of CCVs but also show that the recruitment is impaired if phosphatidylinositol (4,5)bisphosphate, a phosphoinositide highly enriched in plasma membranes, is present on the membrane.
This is a high-quality manuscript that, while confirming many previous observations in the mature Rab5 field, clearly goes beyond the state of the art (as far as I can judge), resulting in a coherent and convincing picture of the recruiting mechanisms with many novel elements. The manuscript contains an impressive amount of data, with hundreds of individual experiments in the main and extended figures. While the overall logical flow is perfectly clear, the MS is not always easy to read, and in some parts I needed to go through text and the corresponding figures several times to understand the experiments (particularly the figure legends). Thus, the manuscript may benefit from some editing to improve clarity. For instance, the standard type of analysis as shown the first time in Fig. 1 a-c (and in extended Fig. 1), needs to be better explained – what are lifetime cohorts, and what do the overlaid curves represent? Why is it important to look at these different cohorts – are there any differences between endocytic events that retain clathrin for shorter or longer time periods? Also, it would help readability if some of the non-textbook-standard abbreviations can be avoided (EE, UEC, CCP), and perhaps some of the constructs may be given a more intelligible abbreviation (such as CLTA-670nano, which is essentially a tagged clathrin light chain).
Aside from these minor comments I do not have any serious critique – this is a well-reasoned study of excellent quality. I do not see any room for shortening – the data both in the main figures and the extended figures are all required to support the conclusions of the manuscript.
Minor technical point:
Extended Fig. 1g: The legend states that there are fluorescence intensity traces of an endocytic event (as in panels d-f), but I could not detect this in the figure.

Reviewer #2

(Remarks to the Author)
The manuscript ‘Lipid turnover and GEF recruitment collectively determine Rab5 recruitment and activation during the first step of early endosome formation’ addresses the central question, which cellular components are required to recruit Rab5 to the nascent uncoated endocytic carrier after loss of the clathrin coat. This work sheds light onto the temporal relationship between different components and their catalytic activity in this process. It identifies Rabex5 and hRME6 as pivotal proteins in this process and, perhaps somewhat unexpected, discover that the lipid composition of the Clathrin coated vesicle additionally prevents early recruitment of Rab5. The hydrolysis of PI(4,5)P2 is prerequisite of Rab5 recruitment and provides an additional regulatory level.

The authors provide high-quality live cell imaging in combination with state-of-the art image analysis. They also confirm and extend their imaging results with biochemical and in vitro experiments providing a comprehensive view of the interactions leading to Rab5 recruitment. The sheer number of experiments and their associated controls makes the manuscript challenging yet enjoyable to read. The authors carefully discuss their results and acknowledge that, despite their extensive efforts, some details remain to be characterised. Overall, this work is a crucial step in our undertsanding of the endocytic process and therefore highly relevant. This systematic study will certainly be a valuable resource and provides novel and important information on clathrin mediated endocytosis and endosome formation. I would like to congratulate the authors on their detailed characterisation and their work towards a comprehensive model. I hope that the authors will continue to fill in the remaining gaps in future publications.

I still would like to see a few points be clarified and / or addressed before publication:

1) It would be valuable information, if there is a correlation between Rab5 recruitment and onset of movement / increased mobility of the vesicle. I would encourage the authors to revisit their TIRF-imaging data along these lines and include this information in the manuscript.

2) Variation in 'Clathrin-density' on the plasma membrane: Clathrin structures larger than a single CCP are visible for example in Movie 1 and Movie 2. These structures display multiple recruitments of Rab5c and Rabex5, respectively. These could resemble stable clathrin structures as described by Merrifield (for example: Merrifield et al, Cell, 2005; Taylor et al, PLos Biol, 2011; Lampe et al., J Struct Biol, 2016). It would be a valuable information, to briefly describe how these regions are treated during the analysis procedure. As the Rab5 recruitment appears after the scission of the vesicle, I would not assume that there should be a difference between budding sites at regions of different clathrin density, but it would be good if the authors could briefly comment on this.

3) Imaging parameters: The authors should provide more details about their imaging parameters. At least typical settings (like laser power (in kW/cm^2), exposure time and major camera settings) as well as typical values for recorded signal intensities should be mentioned supporting the reproducibility of the data. Alternatively or in addition, the authors might want to consider making at least some of their annotated data publicly available via the BioImage Archive or similar databases.

4) Schematic overview (Figure 7c), especially lower part on VPS9: In this cartoon, the authors summarize the essential findings of their work. Adding additional details and improve the description of the figure in the legend would certainly help readers to cope with the vast amount of information provided in the manuscript. I would especially motivate the authors to re-visit the lower part of the figure, which lacks any detailed description in the figure legend and cannot be understood without having a careful read of the paper.
The authors might want to consider amend the figure such, that it can be used as a graphical summary of their work and help guiding readers as well as emphasizing their major findings, e.g. by adding information about catalytic activity etc..

5) Minor detail / ambiguity - Extended Data Fig. 9. 'The failure of Rab5 recruitment to the Vps9- or Rabex5-mistargeted to the plasma membrane is not related to GAP proteins.' Might be changed to (or similar): 'The failure of Rab5 recruitment to Vps9- or Rabex5, which were mistargeted to the plasma membrane, is not related to GAP proteins'?

Reviewer #3

(Remarks to the Author)
The small GTPase Rab5 is well documented for its function in early endosome fusion and endocytosis. This manuscript investigates the molecular mechanisms underlying the spatiotemporal recruitment of Rab5 during early endosome (EE) formation. Using single-molecule microscopy in genome-edited cells, the authors identify two Rab5 guanine-nucleotide exchange factors (GEFs), Rabex5 and hRME6, as key regulators of Rab5 recruitment and activation on nascent uncoated endocytic carriers (UECs). They show that Rabex5 is continuously recruited to UECs, while hRME6 is recruited transiently. Rabex5 knockout significantly reduces Rab5 recruitment and number of EE while Rabex5/hRME6 double knockout essentially abolish this process. In addition, the study makes an novel observation that the plasma membrane–enriched phosphoinositide $PI(4,5)P_2$ plays a negative role in Rab5 recruitment and prevents its association with the plasma membrane. This finding is important as it may imply a general role of organelle-specific phosphoinositides in membrane targeting and activation of Rab GTPases during vesicular transport. Finally, impaired $PI(4,5)P_2$ hydrolysis in Lowe syndrome cells leads to defective Rab5 recruitment and EE formation, linking this mechanism to human disease.
The manuscript addresses a longstanding question in endocytic trafficking and presents novel insights into the spatial regulation of Rab5 membrane recruitment and activation. However, several experimental issues and broader implications require clarification or additional evidence before publication.
Specific Comments:
1. The manuscript relies heavily on the concept of UECs, but their precise molecular identity is not well established: do they represent a distinct compartment? How are they biochemically different from EE? More evidence is needed to establish this concept including specific protein and lipid markers.
2. In addition to Rab5, there are multiple Rabs associated with EE among which Rab21 is also a substrate for Rabex5 and should be included as an important positive control in the Rabex5 knockout experiments. Another EE-associated Rab such as Rab4 or Rab22 that does not respond to Rabex5 may serve as a negative control. Along this line, Rabex5/hRME double knockout cells effectively abolish Rab5-positive EE. Are other EEs, e.g., Rab4 EE or Rab22 EE, affected?
3. In both the Rabex5/hRME knockout and the Lowe syndrome experiments, a rescue control should be included to strengthen authors' claim.

4. Previous studies on Rab5 and Rabex5 should be discussed in more detail to place these findings in context. For example, the first subheading in Results “All three isoforms of Rab5…” is not wrong but imprecise and can be misleading, as the new information is actually for Rab5b while Rab5a and Rab5c results reported previously (ref 15). A more appropriate subheading may be “Rab5b, like Rab5a and Rab5c…”. In addition, the section suggesting “The Vps9 domain is essential for Rabex5 recruitment…) is misleading, as Fig 3 shows that the Vps9 domain alone is insufficient to associate with the membrane, although this Vps9 domain construct is not described in the text. This result and the conclusion that neither the N-terminal UBD nor the C-terminal domain including RpBD is necessary for Rabex5 membrane targeting to EE are consistent with a previous paper addressing Rabex5 membrane targeting domain (ref 22). This and another paper on the same topic (ref 23) are cited but not discussed in the context of new findings.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
During revision the authors have carefully addressed all of my (minor) remarks. Thus, I now recommend publication of this excellent manuscript.

Reviewer #2

(Remarks to the Author)
All the points I raised in my previous comments have been adequately addressed, except for one minor detail. The author should be able to clarify this by re-analysing the existing data with respect to the onset of vesicle mobility after Rab5 recruitment (see Extended Fig. 1d–g):
Not all events appear to have an immediate onset of mobility. For example, Extended Fig. 1f shows a rather stationary event, whereas Figs. 1d and 1e seem to show an immediate onset of mobility upon Rab5 recruitment. Is there a time lag between Rab5 recruitment and the onset of mobility? Or is the onset of mobility strictly linked to Rab5 recruitment, with only a small proportion of vesicles remaining stationary? The authors should briefly comment on this and present adequate data in the form of a histogram displaying the time between Rab5 recruitment and the onset of mobility, or another suitable graph, to address this question.

Reviewer #3

(Remarks to the Author)
The authors have addressed all my concerns.

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Response to the reviewers' comments:

We would like to extend our sincere gratitude to the reviewers for dedicating their valuable time and providing us with insightful comments and valuable suggestions. During the past three months, we have devoted extensive efforts to conducting a series of new experiments to comprehensively address all reviewers' concerns. We do believe that the review process was highly constructive, and the suggested experiments, analyses, and discussions have significantly strengthened our conclusions and improved the work.

These new or updated results are shown in the revised manuscript, as well as in this response letter. We have edited the manuscript text to improve clarity, including the removal of non-textbook-standard abbreviations. These modifications are highlighted in red in the revised manuscript. Detailed point-by-point responses to the reviewers' comments are enumerated below.

Reviewer #1 (Remarks to the Author):

In this study, the authors address the mechanisms by which the small GTPase Rab5 is targeted to endocytosing membranes resulting in the formation of early endosomes. To this aim, the authors use sets of genetically engineered cell lines expressing tagged variants of Rab5 isoforms, various GTP-exchange factors of Rab5 and additional proteins involved in the formation and uncoating of clathrin-coated vesicles to not only arrive at a precise sequence of recruiting but also to pinpoint the recruiting mechanisms. Using advanced and automated time-lapse imaging, the authors not only show that the two GEFs Rabex and hRM6 cooperate to recruit Rab5 immediately after uncoating of CCVs but also show that the recruitment is impaired if phosphatidylinositol (4,5)bisphosphate, a phosphoinositide highly enriched in plasma membranes, is present on the membrane.

This is a high-quality manuscript that, while confirming many previous observations in the mature Rab5 field, clearly goes beyond the state of the art (as far as I can judge), resulting in a coherent and convincing picture of the recruiting mechanisms with many novel elements. The manuscript contains an impressive amount of data, with hundreds of individual experiments in the main and extended figures. While the overall logical flow is perfectly clear, the MS is not always easy to read, and in some parts I needed to go through text and the corresponding figures several times to understand the experiments (particularly the figure legends). Thus, the manuscript may benefit from some editing to improve clarity. For instance, the standard type of analysis as shown the first time in Fig. 1 a-c (and in extended Fig. 1), needs to be better explained – what are lifetime cohorts, and what do the overlaid curves represent? Why is it important to look at these different cohorts – are there any differences between endocytic events that retain clathrin for shorter or longer time periods? Also, it would help readability if some of the non-textbook-standard

abbreviations can be avoided (EE, UEC, CCP), and perhaps some of the constructs may be given a more intelligible abbreviation (such as CLTA-670nano, which is essentially a tagged clathrin light chain).

Aside from these minor comments I do not have any serious critique – this is a well-reasoned study of excellent quality. I do not see any room for shortening – the data both in the main figures and the extended figures are all required to support the conclusions of the manuscript.

Response: We are immensely grateful to the reviewer for the precise summary, positive comments, and very helpful suggestions. We apologize for the lack of clarity in our initial submission and have now extensively edited the manuscript to address your points and improve overall clarity.

Specifically, we have made the following revisions:

(1) Following your suggestion, we have provided a detailed description of the lifetime cohorts in the figure legend for Fig. 1a-c: "*Clathrin-mediated endocytic events with clathrin lifetimes between 20 and 100 s were identified in 31 (**a**), 26 (**b**), or 28 (**c**) cells. To account for the broad distribution of lifetimes, events were grouped into cohorts based on specific clathrin-670nano lifetime ranges. For each cohort, the fluorescence intensity traces for clathrin-670nano (blue), EGFP-Rab5 (green), and mScarlet-I-GAK (red) were averaged and are shown as mean ± SEM (intensity-lifetime cohorts). The number of traces analyzed is shown above each cohort.*"

(2) We have also provided comments on the endocytic events with shorter or longer lifetime in the text as "*Despite the wide range of lifetime distributions of endocytic events, quantitative analysis of the recruitment timing of Rab5a/b/c and GAK in each endocytic vesicle further confirmed that Rab5a/b/c was recruited after the burst-like signal of GAK (Fig. 1a-c)*."

(3) In the revised manuscript, we have replaced these non-textbook-standard abbreviations (e.g. CCP, CCV, UEC, EE) with their full names (clathrin-coated pit, clathrin-coated vesicle, uncoated endocytic carrier, early endosome). We have also defined 'uncoated endocytic carriers' within the text and updated the model in Figure 7c to clearly label these structures, thereby enhancing overall clarity.

(4) We have changed CLTA-670nano to clathrin-670nano, CLTA-TagRFP to clathrin-TagRFP in the revised text and figures.

(5) Additionally, we have added more information regarding the imaging data collection and analysis, results description and discussion, and other editing in the revised manuscript.

Minor technical point:

Extended Fig. 1g: The legend states that there are fluorescence intensity traces of an endocytic event (as in panels d-f), but I could not detect this in the figure.

Response: We thank the reviewer for identifying this error. The figure legend has been corrected by removing "the fluorescence intensity traces of an endocytic event" (**new Extended Fig. 1h**).

Reviewer #2 (Remarks to the Author):

The manuscript 'Lipid turnover and GEF recruitment collectively determine Rab5 recruitment and activation during the first step of early endosome formation' addresses the central question, which cellular components are required to recruit Rab5 to the nascent uncoated endocytic carrier after loss of the clathrin coat. This work sheds light onto the temporal relationship between different components and their catalytic activity in this process. It identifies Rabex5 and hRME6 as pivotal proteins in this process and, perhaps somewhat unexpected, discover that the lipid composition of the Clathrin coated vesicle additionally prevents early recruitment of Rab5. The hydrolysis of PI(4,5)P2 is prerequisite of Rab5 recruitment and provides an additional regulatory level.

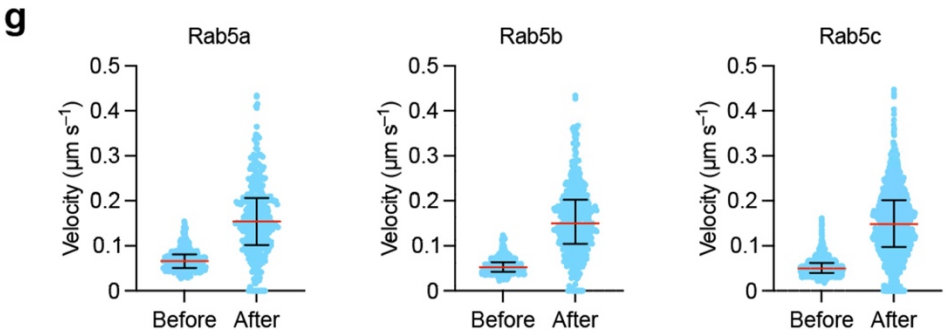
The authors provide high-quality live cell imaging in combination with state-of-the art image analysis. They also confirm and extend their imaging results with biochemical and in vitro experiments providing a comprehensive view of the interactions leading to Rab5 recruitment. The sheer number of experiments and their associated controls makes the manuscript challenging yet enjoyable to read. The authors carefully discuss their results and acknowledge that, despite their extensive efforts, some details remain to be characterised. Overall, this work is a crucial step in our undertsanding of the endocytic process and therefore highly relevant. This systematic study will certainly be a valuable resource and provides novel and important information on clathrin mediated endocytosis and endosome formation. I would like to congratulate the authors on their detailed characterisation and their work towards a comprehensive model. I hope that the authors will continue to fill in the remaining gaps in future publications.

Response: We are deeply grateful to the reviewer for the very precise summary and insightful comments on our work, and we express our sincere gratitude to the reviewer for the highly constructive suggestions. We also thank the reviewer for the recognition of the significance of this study and supportive comments.

I still would like to see a few points be clarified and / or addressed before publication:

1) It would be valuable information, if there is a correlation between Rab5 recruitment and onset of movement / increased mobility of the vesicle. I would encourage the authors to revisit their TIRF-imaging data along these lines and include this information in the manuscript.

Response: We thank the reviewer for the excellent suggestion. As recommended, we have revisited the TIRF-imaging data and analyzed the velocity of endocytic vesicles before and after Rab5 recruitment. Indeed, the vesicles showed increased movement following Rab5 recruitment. The new results have been provided in **Extended Data Fig. S1g** in the revised manuscript (also shown below for convenience).



Extended Data Fig. S1g. SUM159 cells gene-edited for EGFP-Rab5a$^{+/+}$ and clathrin-670nano$^{+/+}$, EGFP-Rab5b$^{+/+}$ and clathrin-670nano$^{+/+}$, or EGFP-Rab5c$^{+/+}$ and clathrin-670nano$^{+/+}$ were transfected with mScarlet-I-GAK and then imaged at the bottom surface using triple-color TIRF microscopy every 1 s for 300 s. The velocity distribution of endocytic carriers before and after recruiting EGFP-Rab5a, EGFP-Rab5b, or EGFP-Rab5c is shown (median with interquartile range).

2) Variation in 'Clathrin-density' on the plasma membrane: Clathrin structures larger than a single CCP are visible for example in Movie 1 and Movie 2. These structures display multiple recruitments of Rab5c and Rabex5, respectively. These could resemble stable clathrin structures as described by Merrifield (for example: Merrifield et al, Cell, 2005; Taylor et al, PLos Biol, 2011; Lampe et al., J Struct Biol, 2016). It would be a valuable information, to briefly describe how these regions are treated during the analysis procedure. As the Rab5 recruitment appears after the scission of the vesicle, I would not assume that there should be a difference between budding sites at regions of different clathrin density, but it would be good if the authors could briefly comment on this.

Response: We thank the reviewer for highlighting this intriguing observation and for the excellent suggestion. Indeed, some larger clathrin structures are present on the bottom surface of SUM159 cells. These structures correspond either to the flat clathrin lattices or 'plaques' described by Merrifield (Lampe et al., 2016; Merrifield et al., 2005; Taylor et al., 2011), or to aggregates of active coated pits as revealed by live-cell structured illumination microscopy imaging (Li et al.,

2015). In our previous study, we observed consecutive bursts of GAK recruitment to these long-lived, larger clathrin structures in gene-edited SUM159 cells expressing AP2-TagRFP$^{+/+}$ (He et al., 2020). As noted, and similar to GAK, some of these larger structures can also recruit multiple rounds of Rab5 and Rabex5. This likely represents the formation of endocytic carriers at the edge of a clathrin plaque or from a cluster of active coated pits.

Since the resolution of our live-cell TIRF imaging was insufficient to resolve individual endocytic events within these larger structures, we excluded these long-lived clathrin assemblies from the analysis. Following the reviewer's suggestion, we have added this clarification to the Methods section and cited the relevant work by Merrifield in the revised manuscript (lines 791-793 on page 27): "*Valid tracks from single clathrin-coated pits were used for further analysis. Persistent tracks and large clathrin structures ('plaques')* [68, 69] *were excluded from this analysis.*" Finally, in our imaging experiments, we did not observe apparent difference among budding sites located in regions with different clathrin densities, which could be an interesting topic for future study.

3) Imaging parameters: The authors should provide more details about their imaging parameters. At least typical settings (like laser power (in kW/cm²), exposure time and major camera settings) as well as typical values for recorded signal intensities should be mentioned supporting the reproducibility of the data. Alternatively or in addition, the authors might want to consider making at least some of their annotated data publicly available via the BioImage Archive or similar databases.

Response: We thank the reviewer for the suggestion. The detailed imaging parameters have now been added to the Method section of the revised manuscript (lines 776-783 on page 27): "*The 1.5× tube lens was applied to achieve the final pixel size corresponding to 86.7 nm of the image. Images were acquired using Micro-Manager 2.0* [65]*. For single-molecular imaging of EGFP/mEGFP-tagged proteins, the 488 nm laser power measured at the objective exit in epi-illumination mode was 1.5 mW. The exposure time was 100 ms per channel, and the EM gain of the EMCCD camera was set to 280 (Evolve 512 Delta) or 300 (iXon Life 888). The imaging interval and duration for each experiment are reported in the respective figure legends. The peak signal-to-noise ratio for purified single mEGFP molecules imaged on coverslips under these single-molecule TIRF conditions was approximately 10.*"

4) Schematic overview (Figure 7c), especially lower part on VPS9: In this cartoon, the authors summarize the essential findings of their work. Adding additional details and improve the description of the figure in the legend would certainly help readers to cope with the vast amount

of information provided in the manuscript. I would especially motivate the authors to re-visit the lower part of the figure, which lacks any detailed description in the figure legend and cannot be understood without having a careful read of the paper. The authors might want to consider amend the figure such, that it can be used as a graphical summary of their work and help guiding readers as well as emphasizing their major findings, e.g. by adding information about catalytic activity etc.

Response: Thank you for the excellent suggestion. We have now revised the schematic overview as well as added a detailed description in the figure legend to improve the clarity (**Figure 7c**) in the revised manuscript (also shown below for convenience).

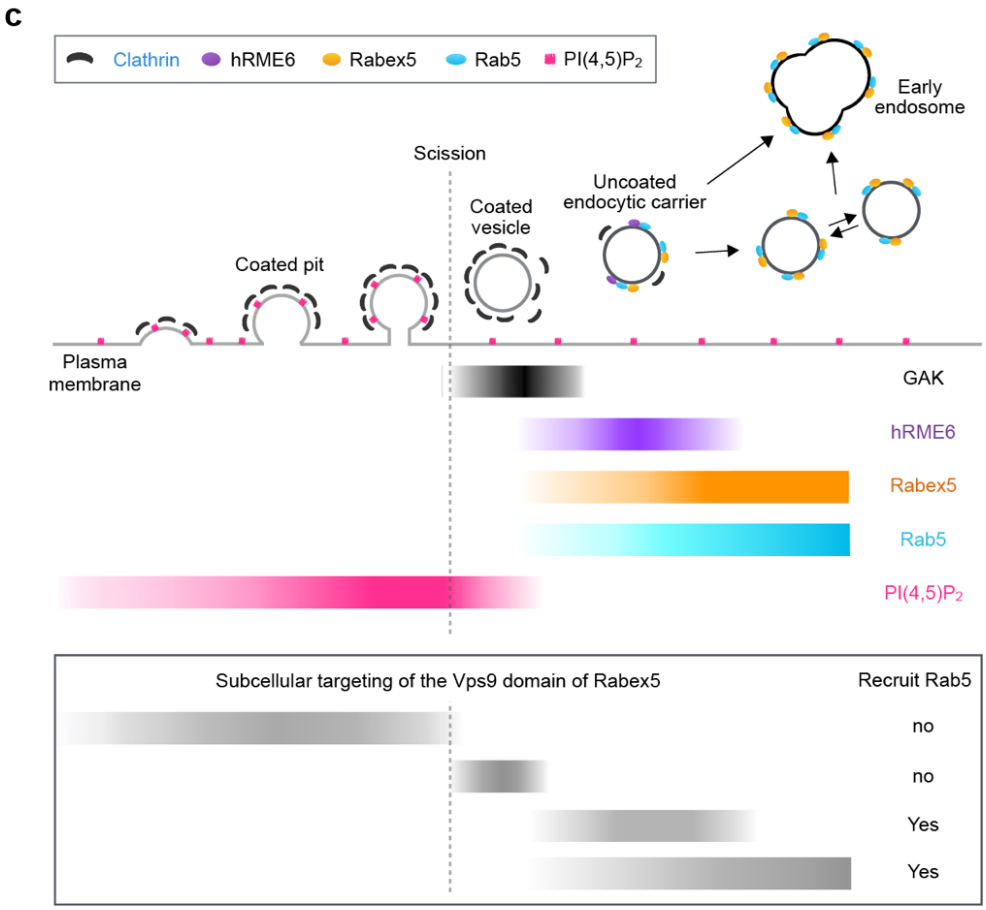


Figure 7c. Schematic summary of the dynamics and regulation of Rab5 during early endosome formation. Clathrin-coated pits form at the $PI(4,5)P_2$-enriched plasma membrane. After scission, endocytic vesicles undergo rapid lipid conversion, including depletion of $PI(4,5)P_2$. Auxilin1/GAK is then recruited in a burst-like manner to the budded coated vesicles to generate the uncoated endocytic carriers. The two GEF molecules, Rabex5 and hRME6, are recruited continuously or transiently following clathrin uncoating. Unlike uncoated endocytic carriers or endosomes, which lack $PI(4,5)P_2$, the mistargeting of Rabex5 or its Vps9 domain to the plasma membrane or coated vesicles fails to recruit Rab5. Thus, $PI(4,5)P_2$ depletion and Rabex5/hRME6 recruitment together determine Rab5 recruitment and activation during the initial stage of early endosome formation.

5) Minor detail / ambiguity - Extended Data Fig. 9. ‘The failure of Rab5 recruitment to the Vps9- or Rabex5-mistargeted to the plasma membrane is not related to GAP proteins.’ Might be changed to (or similar): ‘The failure of Rab5 recruitment to Vps9- or Rabex5, which were mistargeted to the plasma membrane, is not related to GAP proteins’?

Response: We thank the reviewer for the excellent suggestion. As advised, we have revised the sentence to "*Failure of Rab5 recruitment to plasma membrane-targeted Rabex5 or its Vps9 domain is not related to GAP proteins.*".

Reviewer #3 (Remarks to the Author):

The small GTPase Rab5 is well documented for its function in early endosome fusion and endocytosis. This manuscript investigates the molecular mechanisms underlying the spatiotemporal recruitment of Rab5 during early endosome (EE) formation. Using single-molecule microscopy in genome-edited cells, the authors identify two Rab5 guanine-nucleotide exchange factors (GEFs), Rabex5 and hRME6, as key regulators of Rab5 recruitment and activation on nascent uncoated endocytic carriers (UECs). They show that Rabex5 is continuously recruited to UECs, while hRME6 is recruited transiently. Rabex5 knockout significantly reduces Rab5 recruitment and number of EE while Rabex5/hRME6 double knockout essentially abolish this process. In addition, the study makes an novel observation that the plasma membrane–enriched phosphoinositide $PI(4,5)P_2$ plays a negative role in Rab5 recruitment and prevents its association with the plasma membrane. This finding is important as it may imply a general role of organelle-specific phosphoinositides in membrane targeting and activation of Rab GTPases during vesicular transport. Finally, impaired $PI(4,5)P_2$ hydrolysis in Lowe syndrome cells leads to defective Rab5 recruitment and EE formation, linking this mechanism to human disease.

The manuscript addresses a longstanding question in endocytic trafficking and presents novel insights into the spatial regulation of Rab5 membrane recruitment and activation. However, several experimental issues and broader implications require clarification or additional evidence before publication.

Response: We sincerely appreciate the reviewer’s insightful comments and excellent suggestions, which have been instrumental in strengthening our work. In our revised manuscript, we have performed additional experiments, reorganized the data, and revised the text to consolidate our findings and enhance its overall clarity.

Specific Comments:

1. The manuscript relies heavily on the concept of UECs, but their precise molecular identity is not well established: do they represent a distinct compartment? How are they biochemically different from EE? More evidence is needed to establish this concept including specific protein and lipid markers.

Response: Thank you for this excellent comment. Indeed, as the reviewer noted, the uncoated endocytic carriers (UECs) derived directly from endocytic clathrin-coated vesicles represent an intermediate compartment prior to fusion with early endosomes. Canonical early endosomes are positive for Rab5 and its effector protein EEA1, as well as the lipid marker PI3P. As demonstrated in our previous study (He et al., 2017), uncoated endocytic carriers contain $PI(3,4)P_2$, but not PI3P. The $PI(3,4)P_2$-positive uncoated vesicles recruit Rab5 yet lack EEA1. They subsequently fuse with pre-existing Rab5/EEA1/PI3P-positive endosomes.

We have replicated the findings from our earlier work and further verified that endocytic carriers immediately following clathrin uncoating recruit Rab5 but not EEA1 or a PI3P sensor **(Reviewer Figure 1)**. Thus, uncoated endocytic carriers indeed represent the intermediate stage before fusion with early endosomes. In the revised manuscript, we have cited our prior study and included the description of the molecular identity of UECs: "*In our previous work, we have shown that these newly uncoated endocytic carriers contain $PI(3,4)P_2$ and recruit Rab5, yet lack EEA1 and PI3P prior to fusing with endosomes* [15]". Additionally, we have labeled the uncoated endocytic carriers in the updated model and provided a detailed description in the legend for Figure 7c.

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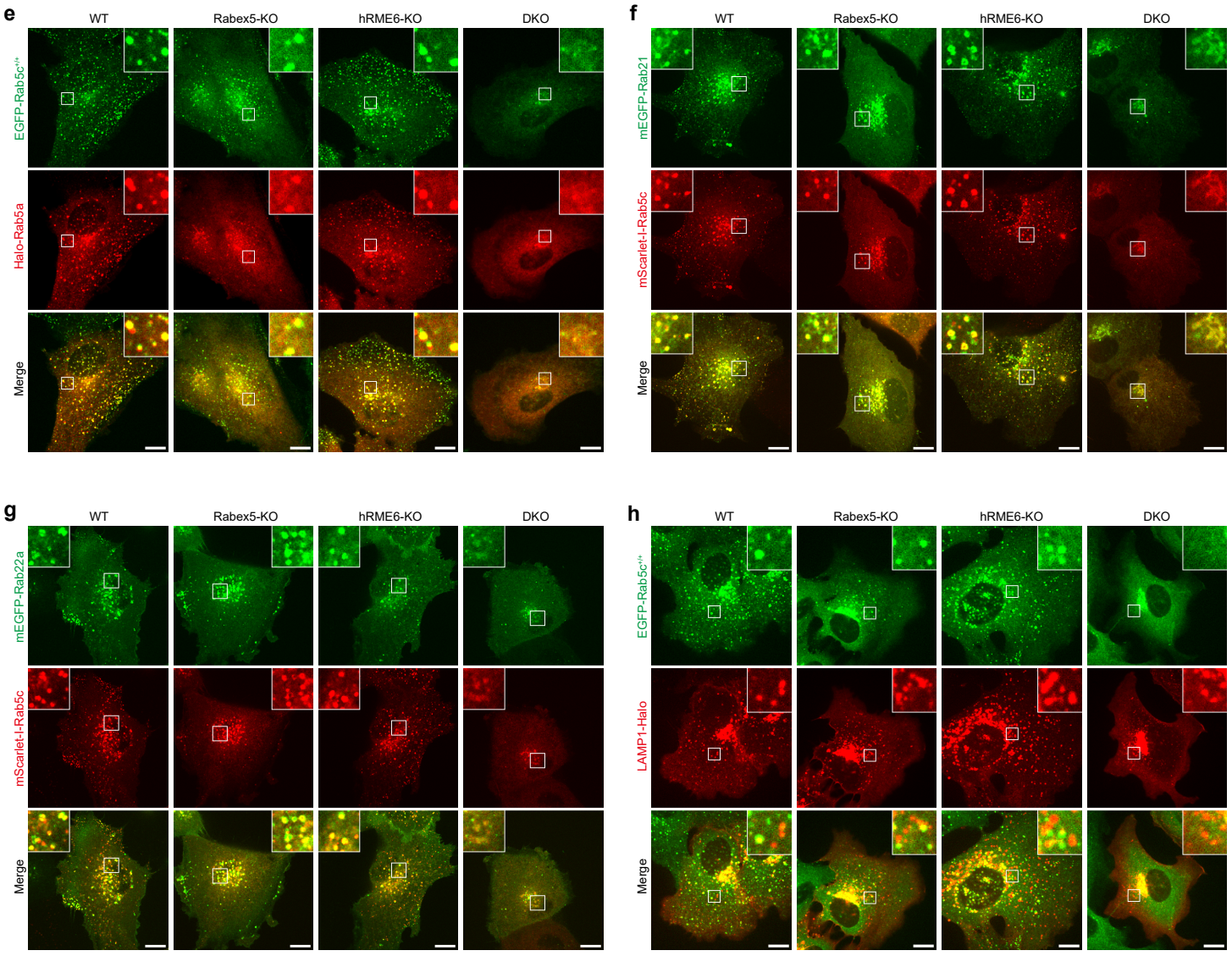
2. In addition to Rab5, there are multiple Rabs associated with EE among which Rab21 is also a substrate for Rabex5 and should be included as an important positive control in the Rabex5 knockout experiments. Another EE-associated Rab such as Rab4 or Rab22 that does not respond to Rabex5 may serve as a negative control. Along this line, Rabex5/hRME double knockout cells effectively abolish Rab5-positive EE. Are other EEs, e.g., Rab4 EE or Rab22 EE, affected?

Response: We thank the reviewer for this excellent suggestion. As advised, we have transiently expressed mEGFP-tagged Rab21, Rab22a, and Rab4a in SUM159 cells with Rabex5-KO, hRME6-KO, or Rabex5/hRME6-DKO and imaged the live cells using spinning-disk confocal microscopy. We found that in Rabex5-KO cells, along with Rab5, the number of Rab21-, Rab22a-, or Rab4a-positive endosomes was decreased. Importantly, and consistent with the loss of Rab5-positive early endosomes, Rabex5/hRME6-DKO resulted in the near-complete disappearance of Rab21- **(new Extended Data Fig. 3f)**, Rab22a- **(new Extended Data Fig. 3g)**, Rab4a-positive endosomes **(Reviewer Figure 2d)**.

Previously, we observed that Rabex5-KO reduces the number of Rab5-positive early endosomes, while Rabex5/hRME6-DKO causes Rab5-positive early endosomes to disappear, leading to the dispersal of other early endosome-associated proteins such as EEA1 **(Fig. 1g)**, APPL1 **(Reviewer Figure 2a)**, Rabaptin5 **(Reviewer Figure 2b),** Rabenosyn-5 **(Reviewer Figure 2c)**, and 2xFYVE(Hrs) **(Fig. 1h)**. Therefore, it is likely that Rabex5/hRME6-DKO causes the loss of Rab5/EEA1-positive early endosomes, which in turn leads to the dispersal of other early endosome-related proteins, including Rab21, Rab22, and Rab4.

The Vps9 domain, together with the HB domain of Rabex5, constitutes the GEF module for the Rab5 subfamily of GTPases, including Rab5 and Rab21. The HB-VPS9 tandem of hRME6 is homologous to the GEF region of Rabex5 (Lodhi et al., 2007). Therefore, whether hRME6 also functions as a GEF for Rab21 remains a subject for future investigation.

These new results and related discussion have been included in the revised manuscript (and below for convenience).



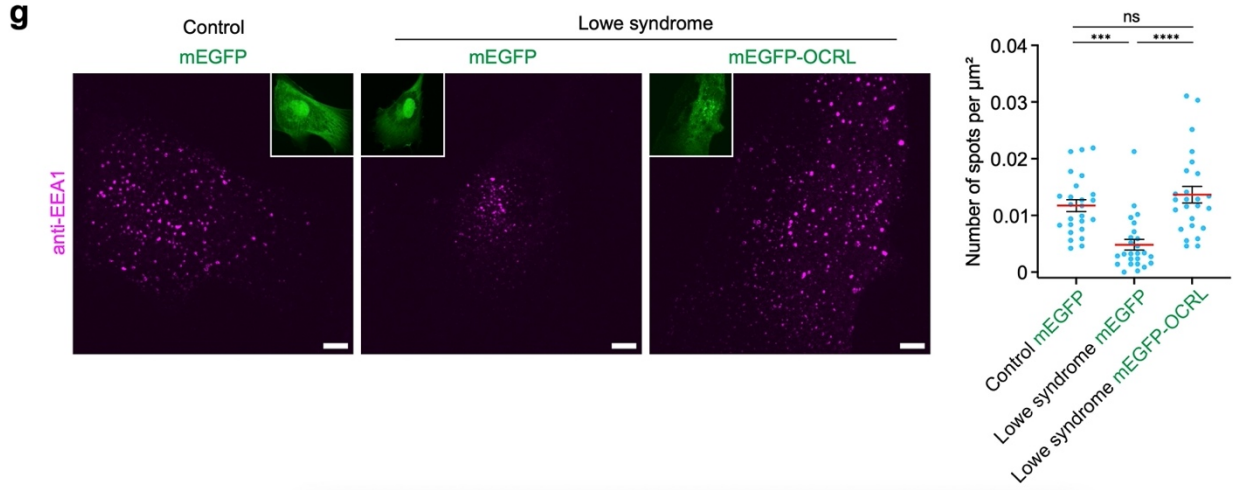
Extended Data Fig. S3f,**g**, SUM159 cells lacking Rabex5 and/or hRME6 expression were transiently transfected with mScarlet-I-Rab5c and mEGFP-Rab21 (**f**), or mScarlet-I-Rab5c and mEGFP-Rab22a (**g**), and then imaged using spinning-disk confocal microscopy. Representative images show the distribution of mScarlet-I-Rab5c with mEGFP-Rab21 or mEGFP-Rab22a around the middle plane of the cells. Scale bars, 10 μm.

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3. In both the Rabex5/hRME knockout and the Lowe syndrome experiments, a rescue control should be included to strengthen authors' claim.

Response: Thank you for the suggestion. In our previous submission, we performed rescue experiments in the Rabex5 and/or hRME knockout SUM159 cells. The re-expression of either full-length Rabex5 (**Fig. 3d, 3e**) or hRME6 (**Fig. 3i, 3j**) was able to restore Rab5 recruitment to uncoated endocytic carriers and early endosome formation.

Following your advice, we have now performed the rescue experiments in Lowe syndrome cells. As expected, transient expression of mEGFP-tagged OCRL restored the formation of EEA1-positive early endosomes. These new results are provided in **Extended Data Fig. S10g** in the revised manuscript (and below for convenience).



Extended Data Fig. S10g, De-identified Lowe syndrome patient fibroblasts and control fibroblasts were transfected with either mEGFP-OCRL or a vector containing mEGFP alone. The cells were then stained for endogenous EEA1 and imaged using spinning-disk confocal microscopy. Numbers of identified EEA1-labeled endosomes are shown on the right (mean ± SEM; 25 cells per group). Statistical analysis was performed using the ordinary one-way ANOVA with Tukey's multiple comparisons test; ***$P < 0.001$; ****$P < 0.0001$; ns, no significance. Scale bars, 10 μm.

4. Previous studies on Rab5 and Rabex5 should be discussed in more detail to place these findings in context. For example, the first subheading in Results "All three isoforms of Rab5…" is not wrong but imprecise and can be misleading, as the new information is actually for Rab5b while Rab5a and Rab5c results reported previously (ref 15). A more appropriate subheading may be "Rab5b, like Rab5a and Rab5c…".

Response: Thank you for the suggestion. In our previous work (ref 15), we generated the gene-edited cells expressing EGFP-tagged Rab5a and Rab5c and performed dual-color TIRF imaging. We observed that Rab5a and Rab5c signals appear after uncoating, as indicated by the disappearance of clathrin signal. In the present study, we have generated new cell lines and extended this analysis by incorporating a GAK signal to mark the precise timing of uncoating. By

per performing triple-color TIRF imaging and quantitative imaging analysis—which was absent from our prior work—we have now verified that Rab5a, Rab5c, and Rab5b are all recruited following GAK-mediated uncoating. Accordingly, we have revised the first subheading in the Results section to "*Rab5 is recruited specifically to uncoated endocytic carriers in genome-edited cells*".

In addition, the section suggesting "The Vps9 domain is essential for Rabex5 recruitment…) is misleading, as Fig 3 shows that the Vps9 domain alone is insufficient to associate with the membrane, although this Vps9 domain construct is not described in the text. This result and the conclusion that neither the N-terminal UBD nor the C-terminal domain including RpBD is necessary for Rabex5 membrane targeting to EE are consistent with a previous paper addressing Rabex5 membrane targeting domain (ref 22). This and another paper on the same topic (ref 23) are cited but not discussed in the context of new findings.

Response: We thank the reviewer for the excellent suggestion. To increase clarity, we have revised the section subheading to "*The Vps9 domain of Rabex5 is necessary for Rabex5 recruitment to uncoated endocytic carriers and Rab5-positive early endosome formation*".

Regarding the results for for UBD and RpBD domains, we have revised the text to "*Consistent with previous studies [22], we observed that removing the N-terminal UBD or the C-terminal RpBD did not affect Rabex5 recruitment to uncoated endocytic carriers*".

References mentioned in this rebuttal document:

He, K., Marsland, R., III, Upadhyayula, S., Song, E., Dang, S., Capraro, B.R., Wang, W., Skillern, W., Gaudin, R., Ma, M.*, et al.* (2017). Dynamics of phosphoinositide conversion in clathrin-mediated endocytic traffic. Nature *552*, 410-414.

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Lampe, M., Vassilopoulos, S., and Merrifield, C. (2016). Clathrin coated pits, plaques and adhesion. J Struct Biol *196*, 48-56.

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Lodhi, I.J., Chiang, S.H., Chang, L., Vollenweider, D., Watson, R.T., Inoue, M., Pessin, J.E., and Saltiel, A.R. (2007). Gapex-5, a Rab31 guanine nucleotide exchange factor that regulates Glut4 trafficking in adipocytes. Cell Metab *5*, 59-72.

Merrifield, C.J., Perrais, D., and Zenisek, D. (2005). Coupling between clathrin-coated-pit invagination, cortactin recruitment, and membrane scission observed in live cells. Cell *121*, 593-606.

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Reviewer #1 (Remarks to the Author):

During revision the authors have carefully addressed all of my (minor) remarks. Thus, I now recommend publication of this excellent manuscript.

Response: We are grateful to the reviewer for the recognition of our work.

Reviewer #2 (Remarks to the Author):

All the points I raised in my previous comments have been adequately addressed, except for one minor detail. The author should be able to clarify this by re-analysing the existing data with respect to the onset of vesicle mobility after Rab5 recruitment (see Extended Fig. 1d–g): Not all events appear to have an immediate onset of mobility. For example, Extended Fig. 1f shows a rather stationary event, whereas Figs. 1d and 1e seem to show an immediate onset of mobility upon Rab5 recruitment. Is there a time lag between Rab5 recruitment and the onset of mobility? Or is the onset of mobility strictly linked to Rab5 recruitment, with only a small proportion of vesicles remaining stationary? The authors should briefly comment on this and present adequate data in the form of a histogram displaying the time between Rab5 recruitment and the onset of mobility, or another suitable graph, to address this question.

Response: We sincerely appreciate the reviewer's recognition of our revised work and for the comments. As suggested by the reviewer during the previous revision, we revisited the TIRF imaging data and calculated the average velocity of endocytic vesicles over periods both before and after Rab5 recruitment. These analyses indicate that vesicle movement generally increases following Rab5 recruitment. However, because the imaging data were acquired at 1-second intervals with single-molecule sensitivity, the temporal resolution was insufficient to reliably calculate transient vesicle velocities using only the frames immediately before and after Rab5 recruitment. Moreover, the data were collected in two dimensions and therefore do not capture the vertical movement of endocytic vesicles. As a result, we were unable to calculate the time lag between Rab5 recruitment and the onset of vesicle mobility based on the available data. Given that this point is not directly relevant to our study and to ensure accuracy, we did not include this result in the revised manuscript.

Reviewer #3 (Remarks to the Author):

The authors have addressed all my concerns.

Response: We are grateful to the reviewer for the recognition of our work.