The Rab11-Family Interacting Proteins reveal selective interaction of mammalian recycling endosomes with the Toxoplasma parasitophorous vacuole in a Rab11- and Arf6-dependent manner

Eric Hartman, Beejan Asady, Julia Romano, and Isabelle Coppens

Corresponding author(s): Isabelle Coppens, Johns Hopkins University

Review Timeline:

| Event                  | Date       |
|------------------------|------------|
| Submission Date        | 2021-06-01 |
| Editorial Decision     | 2021-06-29 |
| Revision Received      | 2022-02-03 |
| Accepted               | 2022-02-23 |

Editor-in-Chief: Matthew Welch

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
RE: Manuscript #E21-06-0284

TITLE: The Rab11-Family Interacting Proteins reveal selective interaction of mammalian recycling endosomes with the Toxoplasma parasitophorous vacuole in a Rab11- and Arf6-dependent manner

Dear Dr. Coppens:

The work is addressing a fascinating biological question although the physiological importance of the recruitment of rab11 or arf6 containing vesicles for parasite fitness is questioned by one of the reviewer.

The reviewers are overall modestly supportive. They identified weaknesses that would need to be addressed in a major revision prior to consideration for publication.

Importantly the manuscript should be better articulated. The objectives and findings need to be more explicit while the discussion is overly long and would greatly benefit to be more concise. Also reviewers 2 and 3 raised some fundamental as well as more technical issues that need to be experimentally addressed.

All the requested experiments are feasible and would consolidate the work and increase its impact.

Sincerely,

Dominique Soldati
Monitoring Editor
Molecular Biology of the Cell

---

Dear Dr. Coppens,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor’s decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor’s and reviewers’ comments have been addressed. (The file type for this letter must be “rebuttal letter”; do not include your response to the Monitoring Editor and reviewers in a “cover letter.”) Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiocell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
Reviewer #1 (Remarks to the Author):

Paper (30112) presents a study of the interaction between mammalian recycling endosomes and Toxoplasma parasitophorous vacuoles (PVs). Using quantitative microscopy, the authors sought to examine the specificity of host Rab vesicle internalization with the PV of Toxoplasma. Overall, the study aim was to uncover a selective process of host recycling endosome recognition and scavenging mediated by Toxoplasma. The paper raised an important question related to specificity of these interactions, as it would help to further elucidate the mechanism of host vesicle internalization and processing within the PV. The paper provided an organized introduction that supported a general understanding of the relationship between the Rab vesicle and PV of Toxoplasma. Furthermore, it also provided a logical rational for the specific focus on the mammalian Rab11 vesicles. The paper seemed to build off of previous findings that showed that Toxoplasma sequesters a broad range of Rab vesicles (especially recycling endosomes) into the PV. The authors did a nice job of including previous findings to support the overall study approach and rationale. The following main study findings were reported: 1) vesicles with FIPs from Class I or Class II were distributed at the PV and detected at varying amounts inside the PV; 2) PV delivery of vesicles with FIPs from Class I was Rab11 dependent; and 3) binding of FIP3 to either Rab11 or Arf6 was found to significantly increase the internalization of vesicles into the PV.

Major Comments:

Introduction.
- Although the paper provides an organized introduction, the potential impact of the study is not as clear. It would be helpful to include (or state more clearly) the significance of the study and how it would add to the field's understanding of host cell invasion and exploitation of membrane trafficking machinery.

Results.
- In Figure-2, micrographs are displayed to show distribution of mammalian vesicles containing FIP. The authors explain that these data are shown in support of demonstrating selectivity. However, it is not clear how these data support the conclusion that Toxoplasma selectively recruits subpopulations of mammalian Rab11 vesicles. It would be interesting to know if the recruitment of the subpopulations of mammalian Rab11 vesicles were truly based on specificity for specific effectors and not just on other factors such as abundance/availability of effectors (i.e. would you still see the same effect if you were to overload the system?). Also, could selectivity be driven by host environment (i.e. are there particular cell signals or effector cell signals during infection that would help enhance selectivity?)

- In Figure-3, it is not clear how the results from the ∆gra2∆gra6 negative control are being interpreted since control % PV with GFP-FIP foci were similar to one of the WT PVs.

Discussion.
- It would be helpful to include a brief summary of the study aims and hypothesis at the beginning of the discussion section to help frame the discussion around key points.

- As written, the discussion section starts with a summary of the main study findings. However, it does not seem to elaborate much on the significance of the different results and overall impact of the study. For example, it would be helpful for the authors to expand upon the point made about subsets of Rab11 vesicles with Class I and Class II FIP being differentially scavenged by Toxoplasma.

- The authors report-"Here we demonstrated the perivacuolar localization of mammalian Arf6 persists throughout infection and that Arf6 vesicles penetrate the PV." However, it is not clear how this point supports the main hypothesis and the overall study context.

- A lot of the discussion section reads more like a review. It would be helpful to incorporate more of the study findings in the discussion to show how specific study results contribute to the narrowing of knowledge gaps relating to Toxoplasma host cell manipulation (how do the specific results further our understanding and the field).

Minor comments:

1. Hypothesis. The hypothesis is not clearly stated.
2. Results. Use of the word "some" in the subheading-Toxoplasma internalizes mammalian Rab11 vesicle subpopulations with either Class I or Class II FIPs into the PV with some selectivity-is vague and uninformative. Perhaps a more accurate description would be varying levels of selectivity.

Reviewer #2 (Remarks to the Author):

This is an interesting manuscript that focuses on understanding for Rab11a/b-endosomes are targeted to the forming PV and how various Rab11-binding proteins may affect this process. The recruitment of endocytic Rabs to PV by Toxoplasma is a fascinating process that remains poorly understood. Thus, this manuscript is timely and addresses a very interesting question. The manuscript does have a few issues that need to be addressed before it is ready for publication (see list below).

1) Antibodies against FIP1, FIP5, FIP2 and FIP3 are now available in various laboratories. Authors should confirm key finding by staining for endogenous FIPs. That is a key experiment since over-expression of FIPs (especially FIP3) does lead to changes in endosome dynamics and sub-cellular localization.

2) Most proposed experiments rely heavily on over-expression of exogenous constructs. While expression of mutants can be informative, they are done in the presence of endogenous wt proteins. Considering that Rab11 and FIPs form hetero-tetramers, that makes interpretation of results more difficult. What happens if authors knock-out or knock-down Rab11a/b or Arf6? Does that affect FIP localization to PV? Similarly, how knock-down or knock-out of various FIPs, especially FIP3 affect Rab11 localization to the PV? siRNAs targeting specific FIPs and Rab11a/b have been published by numerous labs, so it should not be very difficult to do these experiments.

3) RNAseq analysis in Figure 14 is a bit disconnected from rest of the manuscript. Many of the changes are rather small and none of these changes has been validated by qPCR. Finally, it is not at all clear how this RNAseq analysis enhances Rab11 and FIP story presented in Figures 1-13. Thus, in my opinion it should be removed from the manuscript.

Reviewer #3 (Remarks to the Author):

In this manuscript Hartman and colleagues describe the recruitment of rab11, or Arf6 containing vesicles to, and into, the PV of Toxoplasma. A combination of ectopic expression of reporters or antibodies is used to dissect whether different subtypes of rab11 containing vesicles are recruited, which would help to discriminate which host cellular pathways are being co-opted. It is found that rab11 vesicles predominantly interact with Class I FIPs, while Class II FIPs can associate with Arf6, and rab11 respectively. Mutational analysis of FIPs provides some insight into how they associate with the vesicles. This is predominantly a descriptive paper which gives insights into the nature of FIP containing vesicles- but not much experimental evidence for the functional role of the recruitment of the vesicular subsets for Toxoplasma, or the host cell.

The major criticism I would have is that gra2 depleted parasites, which do not accumulate FIP markers of any class tested, have no growth defect in cell culture. It therefore appears as if the cumulative function of the recruitment, has no major impact on parasite fitness. It would be important for the authors to discuss this a bit more in their manuscript. It is also, for some of the markers, the overall amount of the vesicles within the parasite is small. Would that be sufficient for large scale import of nutrients/ alteration of the immune response to occur?

I find overall the paper extensively large, with 14 figure items and a very long discussion with a substantial amount of speculation what the recruitment of the vesicles may be doing to Toxoplasma without experimental evidence. The paper would substantially benefit from a consolidation of the results and the discussion in my opinion.

specific points:

Figure 1A) the RAB11 staining in the uninfected cell looks MUCH more pronounced than in the infected. If the images have been taken and processed with the same parameters, it would mean that there is a substantial down-regulation of rab11, which should be accompanied by Western blots and discussed.

Figure 1C/ D: Comparisons are made between cells with unequal "space" in C, there appear to be 2 nuclei in the cell, confining the space where Rab11B vesicles can be. This could lead to the impression of higher concentration. In D there are 3 vacuoles with an accumulation of vesicles between the three vacuoles, which is maybe misleading. It may be worthwhile to mention in the text, or quantify.

Figure 7: I would like to see a zoomed-out picture of the cells to illustrate that the Abs don't bind everywhere and there is some specificity. It would also help to better line out PVM and PV, as I am not quite clear here what I am looking at. Since the authors use GFP tagged variants, can you use CLEM to better define the vesicles?
RNAseq: The authors perform an RNAseq experiment and observe some modest fold changes for some factors. It would be good if those with the biggest fold changes are probed by Western blot to verify this is a meaningful change on the protein level. Also: there are several datasets out there from infected and uninfected host cells: how does the RNAseq data from this study compare to them?
**Answers to reviewer #1:**

- **Concerning our work hypothesis, significance of the study and advancement in the field:**

  We clarified these points in the Introduction (pages 4 and 5), Discussion (page 13), and Abstract. The aim of our study is to provide further information on how *Toxoplasma* intercepts host Rab11 vesicles by detailing the point of intersection of these vesicles (or subsets of them) with the PV, with the prospect to narrow down the parasite’s needs from host cell recycling pathways. Our data suggest that mammalian Rab11 vesicles are internalized into the PV based on specific Rab11 effectors, which leads us to hypothesize that unique parasite proteins may be involved in the recognition and binding of particular host effectors. Identifying these *Toxoplasma* proteins and examining their potential as drug targets through gene deletion would be our next step.

- **Figure 2: Concerning the selective recruitment of subpopulations of mammalian Rab11 vesicles based on effectors versus abundance in the host cell:**

  As a starting point for our studies, we transfected cells with GFP-FIP constructs which may cause overexpression of FIP and change the FIP vesicle dynamics, as rightly stated by this reviewer. Nevertheless, ever under these conditions, we could observe differential internalization among Rab11-FIP vesicles into the PV. This is more suggestive of a selective recruitment of Rab11 vesicles mediated by specific effector/s than due to different abundance of vesicles in the cell. Besides, no vesicles with Class II FIP with a mutated Rab11-binding domain are observed inside the PV. In the revised version, we tracked the distribution of endogenously produced Rab11-FIP vesicles in infected cells by IFA using anti-FIP3 and FIP5 antibodies (new Figure 9). The percentage of PV containing endogenous FIP3 or FIP5 vesicles and the number of intra-PV FIP3 or FIP5 vesicles are similar to conditions of exogenously expressed GFP-FIP5 or GFP-FIP3 upon transfection. We also demonstrated that silencing Rab11A in infected cells causes a selective reduction in endogenously expressed FIP5 but not FIP3 vesicles, also supporting selective recruitment of Rab11 based on effectors. The possibility that the mammalian vesicle selectivity could be driven by the host environment, like particular cell signals during infection, is a very interesting avenue to explore in the future, more especially as *Toxoplasma* is notorious for secreting proteins that manipulate host cell signaling in its favor.

- **Figure 3: Concerning the interpretation of the results from the Δgra2Δgra6 mutant used as ‘negative’ control:**

  We observed that 0 to 8% PV of Δgra2Δgra6 parasites contain Rab11 vesicles as compared to 12 to 88% PV of wild-type parasites based on intra-PV GFP-FIPs foci (Figure 3). We did not define an arbitrary threshold for a percentage of PV but we added the information that the GFP foci detected in the PV of Δgra2Δgra6 parasites are small based on fluorescence intensity, and often close to the PV membrane, probably encroaching into this membrane. Despite the fact that host organelle internalization is not totally abolished in the PV of Δgra2Δgra6 parasites, we use this mutant as a reference in our assays to compare with wild-type parasites.

**Answers to reviewer #2:**

- **Concerning the confirmation of our data by immunostaining for endogenously expressed FIPs:**

  In the new version, we performed IFA using anti-FIP antibodies for each Class (FIP3 and FIP5) in *Toxoplasma*-infected cells to monitor FIP5 and FIP3 vesicles within the PV (Figures S1 and 9). Our data show comparable values for the percentage of PV containing endogenous FIP3 or FIP5 vesicles and the number of intra-PV FIP3 or FIP5 vesicles to conditions of exogenously expressed GFP-FIP3 or GFP-FIP5 vesicles upon transfection.
- Concerning silencing RNA targeting specific FIPs and Rab11A/B or Arf6:
We thank this reviewer for suggesting downregulation of Rab11 effectors to validate our transfection assays using GFP-FIP constructs (overexpression condition). In new Figure 9, we performed siRNA knockdown for Rab11A in infected cells and monitored FIP3 and FIP5 vesicles within the PV. The aim is to verify our previous findings that vesicles with Class I FIP (e.g., by tracking FIP5) require binding with Rab11 for internalization while vesicles with Class II FIP (e.g., by tracking FIP3) enter the PV regardless of Rab11 interaction (Figures 5 and 6). Under Rab11A depletion, very few PV contained FIP5 foci (90% PV reduction compared to PV in siRNA control cells), with an average of 1.5 foci per PV (compared to 3.5 foci in control PV), confirming the dependence of FIP5 vesicles on Rab11A for PV penetration. No difference was observed for PV with FIP3 vesicles in cells depleted or not for Rab11A, which was expected as the PV internalization of FIP3 vesicles could be mediated by either Rab11 or Arf6 (Figure 13). We did not perform siRNA for Arf6 expression as we would not expect changes in PV sequestration for Class I and Class II FIP vesicles.

- Concerning our RNA sequencing data:
As suggested, we deleted our RNAseq analysis from the manuscript and related points in the Discussion.

---

Answers to reviewer #3:

- Concerning the growth of gra2-depleted parasites and source of nutrients:
Mutants lacking the IntraVacuolar Network (IVN), e.g., ∆gra2 and ∆gra2∆gra6 parasites, have no significant growth defects in cultured cells. In mice, ∆gra2 parasites form less cysts in the brain; no information exists concerning the virulence of ∆gra2∆gra6 parasites in vivo. To examine potential sources of nutrients for these mutants impaired in host organelle uptake, we scrutinized the PV membrane of ∆gra2∆gra6 parasites, and observed long, numerous and stable PV membrane projections (PVMP) that pervade the host cytosol (Romano et al., 2017). These PVMP interact with several host organelles, such as lipid droplets and ER elements. We are currently exploring the possibility that these mutants retrieve their nutrients through the usurpation of Membrane Contact Sites formed between the PV and host organelles, wherein molecules, e.g., lipids, transit.

In general, the scavenging of nutrients is an essential process for an intracellular parasite like Toxoplasma that is auxotrophic for so many metabolites. We then assume that this parasite has evolved redundant salvage mechanisms to survive. Uncovering any of these scavenging processes, even if not obviously critical in vitro, is central understand the parasite’s needs and reveal points of vulnerability.

- Concerning the role of host vesicle internalization into the PV:
The scavenging of host organelles (intact) into the PV would allow the parasite to access bulk molecules present in organelles (see model in Romano et al., 2017). We previously showed that Toxoplasma salvages sphingolipids through the interception of host Golgi vesicles marked with Rab14, Rab30 and Rab43 vesicles (Romano et al., 2013) as mentioned in the Introduction. Additionally, we proposed in the Discussion that the interception of host recycling endosomes to the PV would be to actively obstruct host cell cytokinesis. Indeed, it is known that a high proportion of Toxoplasma-infected cells show blockage in cytokinesis progression. As mammalian Arf6, FIP3 and FIP4 drive Rab11 vesicles to the cleavage furrow for membrane supply, hijacking these vesicles would allow the parasite to counteract host cell cytokinesis. One advantage for Toxoplasma would be to delay the epithelial turnover of infected cells in the host intestine, and thus stabilize its replicative niche in gut cells. We are pursuing these hypotheses.

- Concerning the number of Rab11 vesicles inside the PV-as sufficient or not for large scale import of nutrients or host cell alterations:
We counted ~9 Rab11A vesicles per PV (new Figure 1B, panel b), compared to 2 to 5 Rab11 vesicles marked for a specific FIP (Figure 6). Concerning these vesicle numbers, it is important to mention that: i)
these values are based on snap shots of a PV at a given time, and do not represent absolute numbers of host Rab11 vesicles in the PV; ii) intravacuolar Toxoplasma secrete enzymes, including lipases, making conceivable the processing (degradation) of host vesicles trapped into the PV; iii) Toxoplasma internalizes host Rab vesicles from many different trafficking pathways, at least 15 different Rab vesicles have been detected inside PV (Romano et al., 2017). As a matter of the fact, EM observations on the PV lumen reveal a plethora of vesicular or tubular structures that do not seem to originate from the parasite but rather correspond to material derived from the host cell scavenged by Toxoplasma.

We modified the Discussion to better focus on the parasite needs, however if requested, we will be pleased to further reorganize the content of this section.

- Concerning the quality of images shown in Figure 1 and zoom out-image for EM in Figure 7:
We provide new Figure 1: in Toxoplasma-infected cells, the overall staining for Rab11 in the infected cells based on the fluorescence signal is not reduced but is redistributed and concentrated around the PV. We agree that the previous image in Figure 1A shows two host nuclei in the infected cell, which would raise the concern that Rab11 vesicle concentration at the PV might be due to issue of confined space. This is not the case as we previously showed that the relocalization of mammalian Rab vesicles at the PV occurs already 8 h p.i., and at the time, PV are small containing a single parasite.

We also provide new Figures S3 and S4 showing the cells viewed in Figure 7 at lower magnification. The immunoEM staining for FIP3 is predominantly in mammalian cells and intra-PV vesicles.

- Concerning our RNA sequencing data:
As suggested, we deleted our RNAseq analysis from the manuscript and related points in the Discussion.
Dear Dr. Coppens:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

The authors have adequately addressed the concerns raised by the reviewers and the paper is now acceptable for publication providing a valuable contribution to the field.

Sincerely,
Dominique Soldati
Monitoring Editor
Molecular Biology of the Cell

--

Dear Dr. Coppens:

Congratulations on the acceptance of your manuscript. A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

Would you like to see an image related to your accepted manuscript on the cover of MBoC? Please contact the MBoC Editorial Office at mboc@ascb.org to learn how to submit an image.

Authors of Articles and Brief Communications are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

--

Reviewer #1 (Remarks to the Author):

The authors of paper (E21-06-0284R) provide a thoughtful discussion around the interaction between mammalian recycling endosomes and Toxoplasma parasitophorous vacuoles (PVs). The paper highlights the critical role of Rab11 in a selective process of host recycling endosome recognition and scavenging mediated by Toxoplasma. The paper raises an important question related to specificity of these interactions and helps to further elucidate the mechanism of host vesicle internalization and processing within the PV. The authors also include additional text and figures to help support the critical role of specific Rab11A binding in recognition and internalization. These additions help to further support our understanding of mammalian Rab11 vesicle recognition and scavenging as a selective process, as well as provide evidence of selective internalization of Rab11 into the PV. The revised discussion section includes an analysis of study results and puts forth important points for consideration when thinking of specific Toxoplasma proteins playing a critical role in the recognition and binding of host effectors and their potential as drug targets.
Reviewer #2 (Remarks to the Author):

Investigators have addressed most of my concerns. Thus, in my opinion this manuscript is acceptable for publication at MBoC.

Reviewer #3 (Remarks to the Author):

The authors have addressed my points. Congratulations on a nice cell biological study. It will be great to see how the requirement for hijacking these host components unfolds, especially the cytokinesis defect which remains unexplained.