

Toxoplasma gondii VIP1 mediates parasitophorous vacuole-host endoplasmic reticulum interactions to facilitate parasite development

Corresponding Author: Dr Isabelle Coppens

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Summary:

This manuscript reports on a breakthrough in understanding the MCS between the *T. gondii* PVM and host ER. A combination of analogy with other intracellular pathogens engaging the ER with previous proteomic work led the authors to probe VAPA, VAPB and MOSPD2 on the host side, and subsequently rationalized a secreted parasite protein (signal peptide) with a FFAT domain is likely to engage the ER proteins. Firstly, convincing evidence is presented that on the host ER side VAPA, VAPB and MOSPD2 play a critical role, which engage VIP1 on the parasite side, a gene with FFAT domain mapped here, although not all data is equally strong in presentation. From the data it is clear the interaction is critical, but also reveals additional mechanism contribute to ER-PVM engagement. Secondly, the authors ask why the PVR-ER interaction is critical to the parasite, by combining a triple host KO line with a parasite VIP1-KO line. This shows reveals enlarged acidocalcisomes in the parasite. The discussion nicely considers scenarios (immune response, nutrients, signaling), and concludes that the PVM-ER interaction might be critical for Ca^{2+} homeostasis and/or signaling. Lastly, it is discussed that the identified mechanism applies only to a set of cyst forming Coccidia, not to other ER recruiting Apicomplexa like Plasmodium, which aligns with the need for other players in *T. gondii* as well.

Significance: That *T. gondii* engages the ER has been long known, but the mechanism has remained elusive. This is the first report that convincingly identifies molecular components engaged between the host ER and the PVM. The data also shows that although this is a critical interaction, it is not the only mechanism at play in ER-PVM engagement. Secondly, a combination of assembled tools enables a complete disruption of ER-PVM interaction, which allows interrogation of its function. An enlargement of parasite acidocalcisomes points at Ca^{2+} being a critical molecule exchanged over the ER-PVM MCS. Overall, the report is breakthrough in understanding the MCS between *T. gondii* host ER and PVM, which is a great start and at the same time shows there are additional mechanisms at play.

Rigor: The authors probe the mechanisms from various angles with sufficient power to support statistical significance (although presentation is at times debatable, as pointed out below). However, the assays fall short in a couple of places. Firstly, the Linescan images are very cherry-picked as often the overlay in signals in hER and/or PVM looks good on one side of the vacuole, but not so convincing on the other side, raising a potential question on microscope channel registration vs true biology. Secondly, the authors probe VIP1 localization through GFP and mCherry fusion followed by IFA, rather than autofluorescence. In particular the GFP serum is very aspecific as shown by Western blot, which makes the signals observed in IFA and IEM with this antiserum uncertain. Minor: Figure numbering and reference in the text do not align at several places in the manuscript.

Specific points

1. Fig 2f, 24 hr HeLa panel. It is not clear why the different vacuole sizes were only counted for the DKO with WT parasites, and not for the *dgra2dgra6*. What size vacuoles were counted for the other conditions/panels in Fig 2f? The ANOVA analysis in that case does not seem like a fair comparison, as conditions are different. Please elaborate, as this is critical to interpret whether there is actually a difference or not. A similar comment applies to Ext Data Fig 3c lower panel, where vacuole size is differentiated only in 1 condition.
2. Fig 4b, d. Given the strong aspecific signals seen with the GFP antiserum in the western blot (Ext Data Fig 6c), these data need to be very cautiously interpreted. Moreover, panel C with mCherry was also stained with GFP antiserum, which should not recognize mCherry (to this reviewer's knowledge; if different, please include a western blot as well so specificity can be assessed). Why was no live imaging of GFP or mCherry performed, or fixed samples without GFP or mCherry antiserum, as that should negate any concern on antibody specificity. Alternatively, epitope tags instead of autofluorescent proteins would be much more specific. This set of experiments needs to be firmed up and is in its current form not conclusive and not in support of the localization assignments made based on the presented data.
3. Linescan images throughout. It is really hard to see where in the IFA image the linescan was performed. Sometimes a line is

visible, often not, but the scale is too small to see exactly where in the image the line corresponds to. Since very modest shifts are important to support the conclusions (e.g. Fig 5a; mutant line is not visible, unless one really zooms in dramatically), it is critical to present this better. The position of the lines appear somewhat arbitrarily chosen, and could even be registration issues of microscope channel alignment, as typically one side of the parasite looks different from the other side. The quantification in Fig 5b has no statistical analysis (or support?). Maybe line scans can be quantified for overlap on several spots around the vacuole?

4. Fig 5C. What is ORP1 used as positive control? This is never explained; is it known to interact with VAPA, VAPB and MOSPD2?

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1. Line 129/30 “.. which was also detected on PVM projections (PVMP) extending in the host cytoplasm (Fig. 1b).” Please indicated where the PVMPs are in Fig 1b.
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Reviewer #2

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In this manuscript, Nolan et al. are characterizing the molecular machinery involved in the recruitment of the host ER allowing the proper intracellular development of *Toxoplasma gondii*, the agent of human toxoplasmosis. More precisely, they attempted to decipher how the toxoplasma parasite could modify existing host ER membrane contact sites (MCS) machinery consisting of VAP proteins (VAPA, VAPB, MOSPD2), and recruit it via protein interaction to its parasitophorous vacuole (PV, the parasite niche and host-parasite interface within the host cell). Here they used host cells with either double or triple VAP KO mutants to determine that VAPA and VAPB are required for the early recruitment of the host ER to the PV but are not necessary for later developmental parasite stages (suggesting other tethering proteins that could act at these later timepoints). These effects are worsened in the triple KO involving MOSPD2. In *Typell* parasites (physiological parasite strain), VAPA and B are critical to maintain parasite growth. They then searched for parasite proteins bearing a FFAT domain, which normally is used to interact with VAP proteins. They identified a candidate, TgVIP, a dense granule protein which colocalises with VAPA and VAPB at the PV and confirm its interactions with VAPA and VAPB by immunoprecipitation approach with its predicted FFAT domain. IFA assays confirm partially co-localisation of TgVIP1 with VAPA at the PVM. TgVIP1 disruption only partially reduced ER recruitment at the PVM, and induces the accumulation of acidocalcisome in the parasite. This study is novel and focuses on a topic of particular interest in Apicomplexa but also for host-pathogens interaction and beyond for metabolite trafficking: the role of MCS and Lipid Transfer proteins (LTPs) to maintain parasite (cell) survival. More specifically, the study shed light on an important topic, how are host organelles, and more particularly the ER is recruited by the parasite through MCS and host LTP recruitment. The study is well thought with proper imaging approaches that are convincingly deciphering the timing and molecular actors involved on the host side of ER recruitment. Importantly, the authors have experimentally identified for the first time, one of the parasite protein candidate that is involved in this process of ER recruitment, TgVIP1. The study properly shows that TgVIP1 is indeed involved in the interaction with host VAPs (A, B, MOSPD2) and recruitment of the organelle. However, there are several important point missing and important questions that currently remain unanswered and could be resolved with more experiments. Notably, TgVIP1 is only one of the actor, and is not the one required for initial recruitment. Furthermore, its disruption only partially affect ER recruitment. Finally, the study fails to answer one important question: Why is the parasite recruiting ER? For what function? Is this linked to the role of host LTPs recruited? Else?

Therefore, at this point, the study and the data are only partial and would benefit from major additions and revisions before being considered for acceptance.

Major points:

- What do the ER provide or not to the parasite, especially in the context of TgVIP1 KO, as well as in the context of the DKO, or TKO, which display different phenotype of ER recruitment, and different parasite phenotype. The author is a specialist in imaging and previously used fluorescent substrates to monitor incorporation of metabolite, maybe this could be performed. Without this data, we cannot properly conclude on the role of TgVIP1 as a specific role in the recruitment as suggested by the author
- Since TgVIP1 is one of the recruiter for host ER, likely via interactions with VAPs, why does it only slightly reduce ER recruitment? Could the author find other candidates by running IPs on VAPA, or VAPB as was previously perform for MOSPD2 (Cygan et al.; Ferrel et al.) to identify other protein partner that would allow the binding of the ER
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Reviewer #3

(Remarks to the Author)

In this manuscript, Romano and colleagues investigate the mechanisms of host ER recruitment to the *Toxoplasma gondii* PV. This phenomenon has been described for many decades but the mechanisms underpinning this recruitment of host ER was not known. The most significant findings of the papers are (1) a demonstration that VapA and VapB are required recruitment of host ER. This defect was visualized and quantified using elegant electron microscopy images. (2) A parasite protein, TgVIP1 binds to VapA and VapB. Thus, this study has yielded new insights into the mechanism of PV/host ER association for the first time and is of broad interest.

I have two major critiques of this paper as outlined in detail below:

First, the role of TgVIP1 in ER recruitment is unclear. TgVIP1 knockouts only growth defect, and the authors claim that there is less ER recruitment to the PV in these VIP1 knockout parasites upon DKO infection. As outlined below, this statistical analysis was performed incorrectly, with the 'n' being each cell analyzed, rather than each experimental replicate which brings this conclusion into question.

Second, there is an extensive amount of data in the supplemental materials and the inclusion of data in the main text figures that is somewhat unconvincing. Specifically, the statements about the extent of PV/ER interactions in Fig.1 and 5 that were made based off diffraction limited IFA's that do not have the resolution to visualize membrane contacts. Some of the most interesting data is in the extended data including the CZ1 strain data (extended Fig. 3) and quantitation of the VIP1 knockout phenotypes (extended Fig. 8)

Specific comments:

Fig. 1 extended data Fig. 1: The authors make several statements (listed below) about the extent to which various ER markers colocalize with the PV and PVMP's. However, these statements are not backed up by quantitation or measurements such as colocalization analysis. The authors nicely quantitate the ER recruitment in the EM images. Authors should avoid making firm statements about IFAs without quantitation to back them up.

- Line 131: "This fluorescence pattern for VAPs differed from that of the ER localized protein calnexin." It is not apparent to me from the images presented that that the Vaps are enriched at the PV or are closer PV than the ER marker calnexin
- Line 144: "At 24 h pi, we observed a gathering of host calnexin around the PV and to a lesser extent near PVMP." In this case, lesser extent compared to what? Calnexin at 6 hours or MOSPD2 at 6 hrs?
- Line 179: "Interestingly, we observed an expansion of the IVN tubules in PV of parasites infecting DKO cells." Could the authors include the following: How many vacuoles were analyzed? How many independent experiments? What was the frequency with which they observe PVMPs at the 6 and 24 hour time points?
- Extended Data Figure 2b and C – axis labels on the line scan are small and difficult to read and the y-axis values for the WT and DKO cells are not the same. There is clear variability in the calnexin signal around the PV in both host cell types. The line scan was drawn in a region where the ER signal was higher in the KD than the control, although it does show an off set in the signals. This highlights the difficulty in drawing conclusions from a single line scan.

Data analysis in Fig. 5: A vital piece of data in the paper is that demonstration that the VAPA WT recruits the ER more than the K94DM96D mutant, however the methods for how this analysis was conducted is lacking detail. Authors should detail the following – how many images and independent experiments were scored? How was the amount of sharp signal determined (i.e. – $\frac{1}{2}$ vs. $\frac{1}{4}$ vs. $<\frac{1}{4}$), was this measured or just estimated by eye? Can authors include examples of each phenotype in the supplemental data? It would be more convincing to score this data using EM, as in figure 2, rather than diffraction limited IFA.

Statistical analysis: Fig. 3D, extended data fig 7, extended figure 8: Statistics throughout the paper were performed on an experiment by experiment basis with the "n" for the stats as individual cells. The correct statistically method is for the experimental replicates to be "n" (i.e. n=3 for three independent experiments) as outlined by Lord et al 2020 PMID: 32346721.

Minor comments:

Figures are mis-referenced a number of times including,

Line 230 – This figure is labeled as figure 3 but referred to as figure 5 in the text.

Line 347: Should this read figure 6?

Line 356: should this read extended figure 8?

Line 158 – Missing a closing bracket

Decision Letter:

5th March 2025

*Please ensure you delete the link to your author homepage in this e-mail if you wish to forward it to your co-authors.

Dear Isabelle,

Thank you for your patience while your manuscript "Rewiring existing communication: How *Toxoplasma* usurps mammalian ER Membrane Contact Sites" was under peer-review at Nature Microbiology. It has now been seen by 3 referees, whose expertise and comments you will find at the end of this email. Although they find your work of some potential interest, they have raised a number of concerns that will need to be addressed before we can consider publication of the work in Nature Microbiology.

In particular, referee #1 has some concerns regarding antibody specificity, and says the Linescan images need to be presented better. Referee #2 asks for some further mechanistic studies into how the parasite recruits host organelles. Referee #3 also says the role of TgVIP1 in ER recruitment is unclear, and they have some concerns over statistics.

Should further experimental data allow you to address these criticisms, we would be happy to look at a revised manuscript.

We are committed to providing a fair and constructive peer-review process. Please do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We strongly support public availability of data. Please place the data used in your paper into a public data repository, if one exists, or alternatively, present the data as Source Data or Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. For some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found at <https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data>.

Please include a data availability statement as a separate section after Methods but before references, under the heading "Data Availability". This section should inform readers about the availability of the data used to support the conclusions of your study. This information includes accession codes to public repositories (data banks for protein, DNA or RNA sequences, microarray, proteomics data etc...), references to source data published alongside the paper, unique identifiers such as URLs to data repository entries, or data set DOIs, and any other statement about data availability. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, we also strongly encourage including these in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see: <http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf>

When revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already we suggest that you begin to revise your manuscript so that it conforms to our Article format instructions at <http://www.nature.com/nmicrobiol/info/final-submission>. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

When submitting the revised version of your manuscript, please pay close attention to our [href="https://www.nature.com/nature-portfolio/editorial-policies/image-integrity">Digital Image Integrity Guidelines](https://www.nature.com/nature-portfolio/editorial-policies/image-integrity) and to the following points below:

- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
- that control panels for gels and western blots are appropriately described as loading on sample processing controls
- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

EXTENDED DATA FIGURES

When re-submitting your manuscript, please ensure that any supplementary figures and tables that are crucial to the manuscript's conclusions are converted into Extended Data figures and tables to increase visibility of these data. Extended Data figures and tables are online-only (present in the online PDF and full-text HTML versions of the paper), peer-reviewed display items that provide essential background to the article but are not included in the main article due to space constraints. A maximum of ten Extended Data display items (figures and tables) is permitted.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

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If you wish to submit a suitably revised manuscript we would hope to receive it within 5 months. If you cannot send it within this time, please let us know.

In the meantime we hope that you find our referees' comments helpful.

Yours sincerely,

Reviewer Expertise:

Referee #1: Toxoplasma pathogen-host interaction

Referee #2: Toxoplasma host cell interaction

Referee #3: Electron microscopy

Reviewer Comments:

Reviewer #1 (Remarks to the Author):

Summary:

This manuscript reports on a breakthrough in understanding the MCS between the *T. gondii* PVM and host ER. A combination of analogy with other intracellular pathogens engaging the ER with previous proteomic work led the authors to probe VAPA, VAPB and MOSPD2 on the host side, and subsequently rationalized a secreted parasite protein (signal peptide) with a FFAT domain is likely to engage the ER proteins. Firstly, convincing evidence is presented that on the host ER side VAPA, VAPB and MOSPD2 play a critical role, which engage VIP1 on the parasite side, a gene with FFAT domain mapped here, although not all data is equally strong in presentation. From the data it is clear the interaction is critical, but also reveals additional mechanism contribute to ER-PVM engagement. Secondly, the authors ask why the PVR-ER interaction is critical to the parasite, by combining a triple host KO line with a parasite VIP1-KO line. This shows reveals enlarged acidocalcisomes in the parasite. The discussion nicely considers scenarios (immune response, nutrients, signaling), and concludes that the PVM-ER interaction might be critical for Ca^{2+} homeostasis and/or signaling. Lastly, it is discussed that the identified mechanism applies only to a set of cyst forming Coccidia, not to other ER recruiting Apicomplexa like Plasmodium, which aligns with the need for other players in *T. gondii* as well.

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- Line 144: "At 24 h pi, we observed a gathering of host calnexin around the PV and to a lesser extent near PVMP." In this case, lesser extent compared to what? Calnexin at 6 hours or MOSPD2 at 6 hrs?
- Line 179: "Interestingly, we observed an expansion of the IVN tubules in PV of parasites infecting DKO cells." Could the authors include the following: How many vacuoles were analyzed? How many independent experiments? What was the frequency with which they observe PVMPs at the 6 and 24 hour time points?
- Extended Data Figure 2b and C – axis labels on the line scan are small and difficult to read and the y-axis values for the WT and DKO cells are not the same. There is clear variability in the calnexin signal around the PV in both host cell types. The line scan was drawn in a region where the ER signal was higher in the KD than the control, although it does show an off set in the signals. This highlights the difficulty in drawing conclusions from a single line scan.

Data analysis in Fig. 5: A vital piece of data in the paper is that demonstration that the VAPA WT recruits the ER more than the K94DM96D mutant, however the methods for how this analysis was conducted is lacking detail. Authors should detail the following – how many images and independent experiments were scored? How was the amount of sharp signal determined (i.e. – ½ vs. ¼- ½ vs. <¼), was this measured or just estimated by eye? Can authors include examples of each phenotype in the supplemental data? It would be more convincing to score this data using EM, as in figure 2, rather than diffraction limited IFA.

Statistical analysis: Fig. 3D, extended data fig 7, extended figure 8: Statistics throughout the paper were performed on an experiment by experiment basis with the "n" for the stats as individual cells. The correct statistically method is for the experimental replicates to be "n" (i.e. n=3 for three independent experiments) as outlined by Lord et al 2020 PMID: 32346721.

Minor comments:

Figures are mis-referenced a number of times including,

Line 230 – This figure is labeled as figure 3 but referred to as figure 5 in the text.

Line 347: Should this read figure 6?

Line 356: should this read extended figure 8?

Line 158 – Missing a closing bracket

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have been very responsive to the review. In particular, the added quantifications of signal co-localization have strengthened the manuscript, and overall the presentation of (new) data have improved the manuscripts substantially.

Reviewer #3

(Remarks to the Author)

The authors have adequately addressed all my concerns.

Decision Letter:

Our ref: NMICROBIOL-25010020A

25th July 2025

Dear Isabelle,

Thank you for submitting your revised manuscript "Rewiring existing communication: How Toxoplasma usurps mammalian ER Membrane Contact Sites" (NMICROBIOL-25010020A). It has now been seen by two of the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Microbiology, pending minor revisions to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about two weeks. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Microbiology. Please do not hesitate to contact me if you have any questions.

Sincerely,

Reviewer #1 (Remarks to the Author):

The authors have been very responsive to the review. In particular, the added quantifications of signal co-localization have strengthened the manuscript, and overall the presentation of (new) data have improved the manuscripts substantially.

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed all my concerns.

Version 2:

Decision Letter:

8th September 2025

Dear Isabelle,

I am pleased to accept your Article "Toxoplasma gondii VIP1 mediates parasitophorous vacuole-host endoplasmic reticulum interactions to facilitate parasite development" for publication in Nature Microbiology. Thank you for having chosen to submit your work to us and many congratulations.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Microbiology style. We look particularly carefully at the titles of all papers to ensure that they are relatively brief and understandable.

Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required. Once your paper has been scheduled for online publication, the Nature press office will be in touch to confirm the details.

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Replies to Reviewer #1

We thank this reviewer for appreciating our study that ‘has identified molecular components between the host ER and the PVM’. As many comments were related to the poor resolution of our fluorescence microscopy images, we have repeated most of our fluorescence microscopy assays to provide better images with quantifications in the revised version. We have appreciated also the meticulous editing of our text.

► Specific points

Point 1: Concerning the ER-PV coverage comparing vacuoles at different sizes in DKO cells for WT and Δ gra2 Δ gra6 parasites and statistical analysis for data in Fig. 2F and Ext Data Fig. 3C:

We thank the reviewer for pointing out this issue and we agree. We have amended the graph (Fig. 2F) to compare samples with the same number of parasites in the PV. The one exception is the DKO strain infected with Δ gra2 Δ gra6 parasites that did not have any PV with more than 4 parasites. We have detailed this fact in the results section. We also amended the graph (ex-Ext Data Fig. 3C, now Fig. 3) to provide information on the size of the PV for infected WT cells in addition to infected DKO cells.

Point 2a: Concerning the quality of our Western blots in Ext data Fig. 6C using the GFP antiserum:

Indeed, our original Western blot using GFP antiserum had strong aspecific bands. We have repeated the Western blot with modifications to the processing steps (e.g., decreased primary antibody concentration, modified primary antibody incubation, and changed blocking buffer) for VIP1-mEmerald and included a new Western blot in Ext. Data Fig. 4E. We also thank the reviewer for pointing out our mistake in panel C (new panel H): an anti-mCherry antiserum was used for the mCherry sample and we had erroneously indicated the wrong antibody. This has been corrected.

Point 2b: Concerning imaging of GFP or mCherry signal on live/fixed samples in the absence of antibodies:

We thank the reviewer for suggesting this assay to assess the antibody specificity. We have updated Ext Data Fig. 4G to provide live cell imaging of TgVIP1-mEmerald and in Fig. 5C, we included new imaging of VIP1-mEmerald signal in fixed samples without antibody to inspect the mEmerald signal. In Fig. 5B, we also included a validation of the anti-GFP antibody in immunofluorescence assays by staining HeLa cells infected with parental parasites.

Point 3: Concerning the Linescan images arbitrarily chosen and not quantified:

We thank the reviewer for pointing out issues with the line scans. We have made the following modifications:

1. We have replaced most line scans with measurements of colocalization, either the fluorescence correlation measurements using Pearson’s correlation coefficient (PCC) or co-occurrence measurements using Mander’s coefficient (M2).

- a. We have used PCC and M2 analysis to compare the level of colocalization of the PV (TgGRA7 signal) and host calnexin with that of the VAP family members (VAPA, VAPB and MOSPD2) in new Fig. 1C).

- b. We have replaced the experiment in ex-Fig. 5A and B with a new experiment where we used PCC analysis to compare the level of colocalization of WT and FFAT-binding mutant GFP-VAPA with the PV (new Fig. 1F).

2. We have kept a linescan to analyze host VAPA puncta and VIP1-mCherry signal at the PV, as we were assessing a specific region. We have increased the size of the inset and the thickness of the line used for the line scan analysis for easier viewing (Fig. 5E). We have also changed the LUT of TgVIP1-mCherry from yellow to cyan for easier visibility in the linescan.

Point 4: Concerning the selection of ORP1 as positive control:

We apologize for the oversight. ORP1 (oxysterol-binding protein-related protein 1) has an FFAT domain that is known to interact with the MSP domain present in VAPA, VAPB, and MOSPD2. This information has been included in the legend of Fig. 5 and in the text (P10L18) with a reference.

► Minor points

1. We have included arrows to indicate the PVMP extending in the host cytoplasm.

- 2, 3, 5, 6 and 10. We rectified our mistakes and cited the correct figures in the text.
4. We thank the reviewer for informing us of the discrepancy for the color legend of FFAT scores. We have amended the color legend to match the scores in the Table.
7. We have added in the text that ‘the two smaller bands on gel could correspond to either proteolytically processed, or degradation products’, as suggested by the reviewer.
8. We have made the grammatical correction.
9. We agree with the reviewer that a reduction in plaque numbers also indicates poor extracellular survival (not only an invasion defect), and we have added this information in the text.
-

Replies to Reviewer #2

We thank this reviewer for acknowledging that our study is novel and focuses on a topic not only of particular interest in Apicomplexa but also for host-pathogen interactions and more generally for metabolite trafficking. We also thank the reviewer for providing pertinent comments for pursuit of our studies on host ER-PVM contacts. All these suggestions constitute exciting avenues of research to address the next question: why *Toxoplasma* forms MCS with the host ER. Our current manuscript focuses on how *Toxoplasma* forms MCS with the host ER, as an essential groundwork to ascertain the existence of canonical MCS at the host ER-PV interface. Our effort was devoted to dissecting the MCS machinery, both from the parasite and host cell sides, for MCS establishment at the molecular and cellular levels using different experimental strategies. As a result, we identified a *Toxoplasma* effector with a FFAT motif interacting with the triad: VAPA, VAPB and MOSPD (with different affinities), based on phenotypic observations on several parasite and host mutants. We also provided data comparing two *Toxoplasma* strains differing in host ER recruitment. In summary, we are confident that our manuscript contains significant and substantial information to advance the field, even if the scope is exclusively focused on the identification of MCS effectors.

Although the reviewer did not provide data-specific comments on our manuscript, we will address the major comments:

1. Regarding the suggested experiment to image the transfer of fluorescent substrates: monitoring the trafficking of de-novo produced metabolites or ions from one organelle to another one at MCS is technically not feasible with the current technologies. Our experimental strategy is to identify by microscopy a host LTP or calcium sensor located at the host ER-PV interface, followed by their genetically modification and analysis of phenotypical consequences on parasite viability, organelle content, transcriptome and lipidome. We have already initiated this study by ‘eliminating’ some LTP (e.g., CERT), not detected at the PV. As our EM data reveal an abnormal accumulation of acidocalcisomes upon MCS reduction, we added to our new model a possible calcium exchange between the PV and host ER (new Ext. data Fig.6).
- 2&3. Regarding the identification of the host ER recruiter/s of *Toxoplasma*: in our manuscript, we did not infer that TgVIP1 is the ER recruiter; rather TgVIP1 seems involved in establishing MCS once the host ER is at the PV based on ultrastructural observations that the PV of Δ TgVIP1 is covered by host ER up to 6hpi but shows less ER retained at the PV during replication. The initial recruiter of host ER may or may not be an interactor with host ER-VAP, but for this manuscript we are not planning to identify as it is beyond the scope. Instead, we are currently analyzing the role of other secreted FFAT-containing proteins (listed in our Table in Ext. data Fig. 3) for MCS formation, in types I and II, in the context of another manuscript.

Replies to Reviewer #3:

We thank this reviewer for recognizing that our study has yielded new insights into the mechanism of PV/host ER association for the first time and is of broad interest. We were able to address the main critiques,

i.e., by correcting the description of our statistical analysis to assess a reduction in ER recruitment to the PV of Δ TgVIP1 parasites upon DKO infection, and by providing fluorescence images at higher resolution and with quantifications. As suggested, we have modified the presentation of our manuscript by inserting as main figures some of our data previously in Extended Figures.

► Specific points

Point 1: Concerning the absence of quantification of the calnexin and VAPs recruitment the IFA images:

In the revised version, we have quantified our immunofluorescence images (old and new ones) by providing measurements of colocalization, either fluorescence correlation measurements using Pearson's correlation coefficient (PCC) or co-occurrence measurements using Mander's coefficient (M2) as detailed below:

a. We have used PCC and M2 analysis to compare the level of colocalization of the PV (TgGRA7 signal) and host calnexin with that of the VAP family members (VAPA, VAPB and MOSPD2) using Fiji ImageJ in new Fig. 1C).

b. We have replaced the experiment in ex-Fig. 5A and B with a new experiment where we used PCC analysis to compare the level of colocalization of WT and FFAT-binding mutant GFP-VAPA with the PV (new Fig. 1F).

Point 2a: Concerning the frequency of expanded IVN tubules in the PV of WT parasites infecting DKO cells:

We have observed this phenotype in all PV sections passing through the IVN. We added this information in the text (P6L26-27).

Point 2b: Concerning the lesser extent of host calnexin near PVMP:

We thank the reviewer for pointing out this ambiguity. We have rephrased this section to clearly describe the comparison of overlap between calnexin signal with PVMP versus MOSPD2 signal with PVMP. (P5L29-31).

Point 3: Concerning our analysis to compare VAPA WT vs. VAPA mutant K94DM96D recruitment and our selection of linescans in Ext Data Fig. 2B-C:

We thank the reviewer for this critique. The scoring was, in fact, done by eye. We have replaced this experiment with a new experiment for which we measured the level of fluorescence correlation using Pearson's correlation coefficient (PCC) between the GFP-VAPA signal (either WT or K94DM96D) and the PV (TgGRA7) in Δ VAPA Δ VAPB HeLa cells (not in WT HeLa cells as initially shown) transfected with plasmid containing GFP-VAPA or GFP-VAPA mutant; we show two representative images for each condition (new Fig. 1D and E) and a graph comparing PCC values (new Fig. 1 F). We have removed our data from Ext Data Fig. 2B-C from the revised version.

Point 4: Concerning the statistical analysis for EM images referring to the "n":

We apologize for this confusion and incorrect terminology as we have improperly used 'n' for PV section number instead of independent experiments. We have updated the figure legends to accurately state that we listed the number of PV measured.

► Minor comments

We rectified our mistakes and cited the correct figures in the text. We added the missing bracket.

OUTLINE OF THE CHANGES TO THE MANUSCRIPT

1. **Abstract:** Edit/trim for length
2. **Main:**
 - a. Remove lines 75 to 90 (information on MCS hijacking by bacteria) removed for space reasons; not essential to understanding the paper
 - b. Lines 73-74; modified text so 1 example of a LTP is given instead of two. Neither is essential for understanding the paper.
 - c. Minor edits to sentence structure (for clarity and space reasons)
3. **Figure 1:**
 - a. New experiment (IFA) for uninfected and infected cells immunostained for VAPA, VAPB, MOSPD2 and TgGRA7
 - b. New images in panels A and B
 - c. Measured level of colocalization (PCC and Mander's) (panel C)
 - d. New experiment (transfection/IFA):
 - i. Panel D/E: images of GFP-VAPA WT and K94DK96D
 - ii. Panel E: Measurements of colocalization (PCC)
4. **Figure 2:**
 - a. New graph for HeLa WT or DKO infected with WT or $\Delta gra2\Delta gra6$ parasites (24 h), distinguishing between PV containing 1-2 or 4-8 parasites
5. **New Figure 3:** (old Extended Data Fig. 3)
 - a. graph in panel C was changed - the size of the PV was detailed as (4-8 μ m and <4 μ m)
6. **Figure 4:** old Figure 3 now named Fig. 4; no other changes
7. **New Figure 5:** combination of old Fig. 4 and 5 with additional changes
 - a. Kept old Fig. 4 panel A (TgVIP1 cartoon of motifs)
 - b. Replaced the TgVIP1-mEmerald IFA with new images from a new experiment
 - i. Panel B: to validate the anti-GFP antibody, HeLa cells infected with parental parasites were immunostained with anti-GFP
 - ii. Panel C: IFA of HeLa infected with TgVIP1-mEmerald and immunostained with anti-GFP; HeLa infected with TgVIP1-mEmerald, native signal with no immunostaining
 - c. Moved panel C to new Extended Data Fig. 4 (panel H)
 - d. Kept old Fig. 4 panel D (IEM of TgVIP1-mEmerald)
 - e. Moved old Fig. 5 panel E to New Fig. 5 (only kept 1 example for space constraints; change the color of TgVIP1-mEmerald from yellow to cyan for greater visibility in the line scan)
 - f. Moved old Fig. 5 panel C to New Fig. 5 panel F (no other changes)
 - g. Removed old Fig. 5 panels A and B; replaced this experiment with a new one to measure the localization of GFP-VAPA WT or FFAT-binding mutant to the PV; (Used DKO cells instead of HeLa WT; measured PCC instead of visual scoring) – new experiment is in Fig. 1 panels D-E
8. **New Figure 6:**
 - a. combined old Fig. 6 with old Extended Data Fig. 8 panels A and B;
 - b. clarified the statistics for new panel B
9. **New Extended Data Figure 1:**

- a. combined old Extended Data Figs. 1 and 2;
- b. removed old Fig. 2 panels B and C (detecting calnexin at the PV in HeLa WT and DKO cells, since the EM data (new Fig. 2) is more precise and accurate)
- 10. New Extended Data Figure 2:** old Extended Data Fig. 4 (no change)
- 11. New Extended Data Figure 3:** old Extended Data 5;
 - a. modified colors of the scoring matrix to match the ones used in the table
- 12. New Extended Data Fig. 4:** old Extended Data Fig. 6;
 - a. rearranged the panels to match the order in the results;
 - b. replaced the TgVIP1-mEmerald western blot with a new one;
 - c. added live cell images of intracellular TgVIP1-mEmerald (new panel G);
 - d. moved the images of TgVIP1-mCherry from old Fig 4 to panel H
- 13. New Extended Data Fig. 5:** old Extended Data Fig. 7; no changes
- 14. New Extended Data Fig. 6:** old Extended Data Fig. 8;
 - a. old panels A and B of Ext Data Fig. 8 moved to new Fig 6;
 - b. modification to the model include adding the STIM1 Ca^{2+} sensor and a putative parasite Ca^{2+} channel or sensor on the PVM.
- 15. Results:** (new additions written in blue)
- 16. Discussion:**
 - a. minor modifications of lines 394 to 414, edited for length and clarity
 - b. lines 415-428: significantly shortened, some information moved to the results and figure legends
 - c. deleted lines 430-436 for space reasons; this paragraph detailed a function of Legionella-hER that is most likely not used by Toxoplasma due to differences in invasion techniques.
 - d. Lines 437-493, edited for length and clarity
- 17. Materials and Methods:** Updates
 - a. mammalian cell transfection updated to include new details
 - b. deleted reference to the Zeiss AxioImager M2 microscope and Volocity software (acquisition and processing) as the corresponding images were replaced with new images using the Leica DMI8 Thunder and Fiji ImageJ
 - c. added methods for live cell imaging and photobleaching correction
 - d. added image analysis details for measurements of PCC and M2
- 18. Figure Legends:**
 - a. Updated figure legends to include new experimental detail
 - b. Updated figure legends to match the rearrangement of figures
 - c. Corrected the incorrect use of “n” and specified that the numbers pertained to number of PV measured.
- 19. References:**
 - a. Added references related to the new experiments