Title:	Intersection of Endocytic and Exocytic Systems in *Toxoplasma gondii*
Authors:	Olivia L. McGovern, Yolanda Rivera-Cuevas, Geetha Kannan, Andrew Narwold Jr., and Vern B. Carruthers
Article Type:	Original Research

Monitoring Editor	Dominique Soldati-Favre
Date Submitted	20 October 2017
Date for Decision 1	4 December 2017
Date Resubmitted	18 January 2018
Date for Decision 2	4 February 2018
Accepted	10 February 2018

Decision and Reviews

Dear Dr. Carruthers, dear Vern,

Thank you for submitting your manuscript "Intersection of endocytic and exocytic systems in Toxoplasma gondii" for consideration for publication in Traffic. I asked two colleagues who are experts in the field to review the paper and their verbatim comments are appended below. I share the view of the referees that the work presented in this paper is of a high technical caliber and will be of interest. However, I also agree with both referees that some mechanistic insight (functional interference of endocytosis) would significantly increase the impact of this study. The referees have made excellent suggestions for the experiments to include.

Although I cannot accept your manuscript for publication at this point, I believe that you will be able to address the referees' concerns and I look forward to receiving your revised manuscript. To expedite handling when you resubmit please be sure to include a response outlining how you have addressed each of the referees' concerns.

Sincerely,

Dominique Soldati-Favre, Ph.D.
Associate Editor

Referee's Comments to the Authors

Referee: 1

Comments to the Author
Review of McGovern et al.,
Summary:
In this study the authors investigate the trafficking of ingested host cell proteins by the apicomplexan parasite Toxoplasma gondii. Previous studies by the Carruther's group demonstrated the intracellular parasites are capable to take up host proteins that are trafficked to a lysosome like compartment, the VAC. However, to date it is unknown, how this protein uptake occurs, which routes are taken and which trafficking factors are used.
In this study the Carruther's group demonstrate that endocytic and exocytic pathways intersect and that the host proteins are delivered to the VAC using a (probably) conserved transport pathway.
Furthermore, the authors present evidences that de novo synthesis and transport of micronemal proteins occur throughout the cell cycle, while rhoptry proteins appear to be synthesised only during S and M/C phase.
Together this study provides important novel insights about the general organisation of endocytic and exocytic transport pathways in Toxoplasma gondii

Own opinion:
The authors use a nice and reliable assay in order to detect and follow the uptake of a reporter protein (mCherry), expressed in the host cell, into the parasite. Previously the authors demonstrated uptake and delivery of the proteins into the VAC, indicating involvement of a “conserved” trafficking machinery, similar to other eukaryotes.
In general, the performed analysis is well conducted and the conclusions appear valid. I think the study is of general interest and can be published after (minor) revision.

Major criticism:
1.) The study is observational and could be substantially improved by providing some mechanism, to determine if ingestions uses (as proposed) conserved factors. This could be achieved by using some mutants for the discussed endocytic factors (Clathrin, Dynamin, etc.). Alternatively, inhibitors such as Pitstop, Phenylarsine or Cytochalasin D could be used to assess if uptake is clathrin or actin dependent.

2.) The whole experimental approach is based on end point assays followed by colocalisation analysis. Although properly done with all necessary controls provided, it would be great if the authors could provide more dynamic insights using time lapse imaging.

3.) The necessity to scratch syringe the parasite prior to image analysis is introducing some uncertainty. Did the authors test if parasites positive for mCherry are viable and can re-invade?

4.) The authors provide data concerning de novo synthesis of microneme and rhoptry proteins and reach the conclusion that micronemes are synthesised throughout the cell cycle, whereas rhoptries are only synthesised during S and M/C phase. These results are in conflict to Besterio et al, 2009 (see Figure 4). In this study the authors concluded that synthesis of micronemes and rhoptries is tightly regulated and asynchronous. Importantly, co-staining with proM2AP/proRon4 or proRon8/proMIC3 demonstrated the presence of PVs with parasites either positive for the rhoptry OR the microneme marker, indicating tight regulation of both. It might be a choice of markers and/or antibodies that leads to these differences. It is suggested to repeat the experiment to solve this issue, since it is a major point of the study.

Minor criticism:
1.) The model in Figure 5 assumes uptake via the micropore. The authors demonstrated in a previous report that the presence of the intravacuolar network is critical for uptake, suggesting that proteins enter the parasite via this network. The authors should discuss this in more detail. At this point it is still unclear how the host proteins enter the parasite.

2.) The discussion could be significantly shortened and more focused on the presented results.

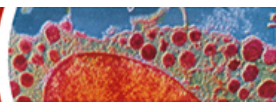
Referee: 2

Comments to the Author
This manuscript provides a careful quantitative imaging-based analysis of the interaction between the newly described endocytic pathway and the better studied exocytic pathway in Toxoplasma. The data generally appears high quality, largely reproducible (although with large error bars in some cases) and is carefully controlled. The data concludes, from a battery of co-localisation experiments, that micronemal proteins and the endocytic pathway intersect, but that rhoptry proteins take a different route and are more tightly cell-cycle regulated.

Overall, I find this work solid and important to report (provided the comments below), however, this manuscript has nothing in the way of functional work to back up the claims of this proposed pathway. This is eminently achievable given available genetic tools and prior knowledge in Rab biology and characterization of molecular players in the Toxo secretory pathway. Any molecular understanding of this pathway would be very welcome and make much more compelling study.

The only major technical point that needs clarification is whether deconvolution was used throughout the study, before co-localisation was scored. This would be essential to discount co-localisation based on detection of out-of-focus light. I cannot find any evidence in the material and methods section. There is only explicit use of deconvolution in Fig S3 and associated text, making me wonder whether it was used throughout or just there?

Minor points:



In the first results section 'Characterisation of parasites stably expressing GalNAc-YFP' – no figure is referenced. I assume the authors should have referred to Figure S1 and S2?

It seems an important observation that GalNac-YFP does probably not exclusively mark the Golgi to to its co-localisation with CPL/B and mCherry. Would it be worth confirming this using the GRASP55 FP fusion?

I find it a little puzzling as to why only 5-15% of parasites (with and without LHVS) have undergone mCherry ingestion. I would have expected higher levels, over the longer periods of time assessed.

Author Rebuttal

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Response
This is a valid and important consideration for the study that we were aware of prior to submitting the manuscript. Accordingly, we had already started to test the role of Dynamin-related protein B (DrpB) in trafficking of ingested material using a regulatable dominant negative mutant of DrpB (ddGFP –DrpB 72A) that was previously shown to impact the trafficking of proteins to the micronemes and rhoptries. Although using a DN mutant is not without it own limitations, we felt this approach was better than using inhibitors that have not been validated in T. gondii and/or that will also have potentially confounding effects on host cells. We also carefully assessed the time-dependent effects of the DN mutant and measured impact at the shortest effective induction times to minimize secondary effects.

Interestingly, as shown in a new 9-panel figure (Figure 7), we found that interfering with DrpB function increased ingestion of host-derived protein into the parasite. The findings imply not only that ingestion does not require DrpB, but also that DrpB might restrict ingestion or that crosstalk between DrpB-dependent and DrpB-independent pathways exists. Possible explanations for these findings are also considered in the revised discussion.

Experiments are planned to test the roles of additional trafficking determinants (clathrin, rabs, adaptor proteins, etc) using the auxin-inducible degradation system, but it will take some time to optimize the system. We envision that such studies will be part of the next "chapter" in this line of investigation.

2.) The whole experimental approach is based on end point assays followed by colocalisation analysis. Although properly done with all necessary controls provided, it would be great if the authors could provide more dynamic insights using time-lapse imaging.

Response
We agree that it would be ideal to visualize ingestion via time-lapse imaging in live, infected cells. However, bright signal from the cytosol of transfected host cells obscures signal within the parasite when visualized by wide field microscopy. We attempted several strategies to obviate this. First, we tried line-scanning confocal microscopy to limit interference from out of focus signal from the host cell cytosol. Second, we used dye called pHrodo-AM, which increases in fluorescence intensity at low pH, to label the host cytosol instead of mCherry. We expected that endocytic delivery of pHrodo to the VAC should result in increased fluorescence intensity due to the acidic environment, thus allowing visualization of ingested material. Unfortunately, neither of these approaches worked sufficiently well to reliably visualize ingestion in live infected cells. We also tried expressing in the host cytosol a photoactivatable form of mCherry (PA-mCh) fused the RAH domain of TgROP5, which directs association with the PVM. This approach significantly reduced signal from the host cell by selectively photoactivating PA-mCh proximal to the PVM. We were able to visualize what appeared to be vesicular trafficking within the PV, but we were unable to track it into the parasite. We also failed to detect accumulation of TgROP5 RAH domain fused to YFP in the parasite using our traditional ingestion assay, implying that ingestion of this protein is below the limit of detection. We are currently testing a protease-activated mCherry, but have thus far not seen evidence of this working. We will continue to invest in strategies for live imaging, but a solution for this is not available presently.

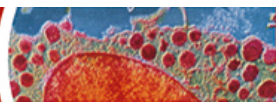
3.) The necessity to scratch syringe the parasite prior to image analysis is introducing some uncertainty. Did the authors test if parasites positive for mCherry are viable and can re-invade?

Response

This is an important point that we tested by recovering LHVS treated parasites from inducible mCherry CHO host cells according to our normal harvest procedure, and inoculated them into fresh HFF cells. We found that mCherry+ parasites were capable of binding to HFF cells and a similar proportion were able to successfully invade host cells (FigureS3A-C), suggesting that mCherry+ positive parasites are viable. That this proportion is somewhat lower than the ~42% of total parasites that were mCherry+ (based on FigureS5C) is probably due to the known effect of LHVS treatment on microneme secretion, which is required for parasite attachment and invasion (Teo et al, Antimicrob Agents Chemother 2007). The ability of mCherry+ parasites to reinvade host cells is now mentioned in the first paragraph of the section titled “Ingested proteins traverse ELCs but likely not the TGN”.

4.) The authors provide data concerning de novo synthesis of microneme and rhoptry proteins and reach the conclusion that micronemes are synthesized throughout the cell cycle, whereas rhoptries are only synthesized during S and M/C phase. These results are in conflict to Besterio et al, 2009 (see Figure 4). In this study the authors concluded that synthesis of micronemes and rhoptries is tightly regulated and asynchronous. Importantly, co-staining with proM2AP/proRon4 or proRon8/proMIC3 demonstrated the presence of PVs with parasites either positive for the rhoptry OR the microneme marker, indicating tight regulation of both. It might be a choice of markers and/or antibodies that leads to these differences. It is suggested to repeat the experiment to solve this issue, since it is a major point of the study.

Response
Although we can understand why the reviewer suggests a conflict between the data in our study versus that of Besteiro et al 2009, our study did not directly test the synchronicity of microneme versus rhoptry synthesis, and the Besteiro study did not assess microneme and rhoptry biogenesis with markers of the cell cycle. Three different individuals in our lab independently obtained similar results for analysis of cell cycle dependent expression of proMICs and proROPs, and, knowing Dr. Besteiro, it can be expected that his analysis was equally thorough. Also, we have previously validated the specificity of the affinity purified anti-proM2AP antibody on Δm2ap mutant parasites. Since we used a new batch of anti-proMIC5 for the current study, we also authenticated its specificity on Δmic5 parasites. To explain the respective findings, we suggest in the discussion that microneme biogenesis could occur in multiple waves during the cell cycle with a pause during a portion of S or M/C phase for rhoptry production. See discussion section “Cell Cycle Dependence of Microneme and Rhoptry Biogenesis”.



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Response
These are both excellent points. Although the micropore is our best guess for the site of endocytosis in T. gondii, there is no direct evidence for this. Accordingly, we removed the micropore from the illustrated model in Figure 8. We also modified the discussion to indicate that the micropore is a likely, but not definitive site of endocytosis, based on the Plasmodium paradigm.

Discussion of the role of the IVN has also been added. As now indicated in the manuscript, Dou et al. (mBio 2014) found that ingestion was reduced in GRA2 knockout parasites, which do not have IVN tubules. This was interpreted to mean that the IVN was required for ingestion, and may contribute as a direct conduit for uptake of host cytosol. Within our model, this would mean endocytosis of IVN tubules into the parasite. However the experiment in Dou et al was performed at 24 h post-infection when GRA2 knockout leads to the loss of IVN tubules and disorganization of parasites within the PV. Our result in Figure 2 that ingestion is active at 7 min post-invasion when IVN tubules have not yet formed, suggests that the IVN is not required for acquisition of host cytosol, at least at this early time point. We have confirmed this by comparing ingestion in WT versus GRA2 knockout parasites at early time points before disorganization occurs (7 min to 3 h post-invasion), and found no significant differences. This data is not included in this paper, and will be part of the next "chapter" of our ongoing studies exploring the mechanisms of ingested protein trafficking across the PVM. With these findings, it remains possible that the IVN contributes to ingestion at later time points, or that the IVN is not directly involved in ingestion but instead affects ingestion indirectly via its role in organizing the parasites within the PV.

2.) The discussion could be significantly shortened and more focused on the presented results.

Response
We have shorted the discussion by ~22% along with placing greater emphasis on the presented results.

Additional changes to the revised manuscript:
Efforts were made to condense all sections of the manuscript. We also assessed the localization of ddGFP-DrpB WT within the T. gondii endolysosomal system (FigureS2) and colocalization of ingested host mCherry with ddGFP-DrpB DrpB (Figure 1) to compliment the data in Figure 7 addressing the role of DrpB in T. gondii endocytosis. Figures S3 and S4 were combined to accommodate a new Figure S2.

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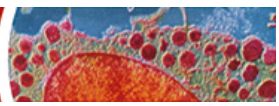
Response
Since this a similar comment was provided by R1, we reiterate the response as follows:



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Interestingly, as shown in a new 9-panel figure (Figure 7), we found that interfering with DrpB function increased ingestion of host-derived protein into the parasite. The findings imply not only that ingestion does not require DrpB, but also that DrpB might restrict ingestion or that crosstalk between DrpB-dependent and DrpB-independent pathways exists. Possible explanations for these findings are also considered in the revised discussion.

Experiments are planned to test the roles of additional trafficking determinants (clathrin, rabs, adaptor proteins, etc) using the auxin-inducible degradation system, but it will take some time to optimize the system. We envision that such studies will be part of the next "chapter" in this line of investigation.

2.) The only major technical point that needs clarification is whether deconvolution was used throughout the study, before co-localisation was scored. This would be essential to discount co-localisation based on detection of out-of-focus light. I cannot find any evidence in the material and methods section. There is only explicit use of deconvolution in FigureS3 and associated text, making me wonder whether it was used throughout or just there?

Response
Deconvolution was used for FigureS4, but it was not used for colocalization studies because we do not have access to a fast deconvolution instrument capable of rapidly capturing the Z-stacks needed for accurate deconvolution on large enough image sets to obtain robust quantitative data. We understand that wide field microscopy has limitations; however, it should be noted that ingested material did not substantially colocalize with all discrete apical markers including the apicoplast or NHE3, providing a measure of validity to the more obvious colocalization seen with proM2AP, CPB, and DrpB (Figure1). We expect that our future work will take advantage of major instrumentation upgrades that are pending at our host institution to achieve greater resolution and thus more precise localization analysis.

Minor points:

1.) In the first results section 'Characterization of parasites stably expressing GalNAc-YFP' – no figure is referenced. I assume the authors should have referred to Figure S1 and S2?

Response
Reference to FigureS1 was inserted into this section.

2.) It seems an important observation that GalNac-YFP does probably not exclusively mark the Golgi to its co-localisation with CPL/B and mCherry. Would it be worth confirming this using the GRASP55 FP fusion?

Response
Thank you for this suggestion. Please see FigureS1 where images comparing GalNac-YFP and GRASP55-RFP are included. In the bottom panel, a new image, which has clearer GRASP55-free signal, has been substituted and white arrowheads indicate the Golgi-free bodies. Please find a description of the colocalization of GalNac-YFP and GRAS55-RFP including a more explicit statement about GalNac-YFP not being restricted to the TGN included in the text results section now titled "Localization of TGN/ELC markers: GalNac and DrpB" and summarized below:

"Consistent with TGN localization, GalNac-YFP appeared in a centrally located structure that overlapped substantially with, or was just apical to, the Golgi marker GRASP55-mRFP (Figure S1). As previously observed, GalNac-YFP sometimes showed a high degree of overlap with another TGN marker the dynamin-related protein DrpB (Figure S1A top panel), but in other cases showed partial or no overlap with DrpB (Figure S1A bottom panel). Interestingly, GalNac-YFP overlapped best with NHE3 in the central region of the parasite despite previous observations that NHE3 partially colocalized with the VAC.36 Therefore, we interpret NHE3 to be an ELC marker that partially overlaps with the TGN, similar to the established ELC marker proM2AP.10,37 GalNac-YFP also partially overlapped with proM2AP,

but rarely colocalized with the VAC markers CPB and CPL or the apicoplast. We also observed that some parasites had GalNac-YFP-labeled structures that were not associated with GRASP55-mRFP (Figure S1, white arrowheads). These structures are reminiscent of "Golgi-free" TGN bodies in plants38 This observation together with substantial overlap with the ELC marker proM2AP, suggests GalNac-YFP occupies the TGN, ELCs, and perhaps additional sites."

3.) I find it a little puzzling as to why only 5-15% of parasites (with and without LHVS) have undergone mCherry ingestion. I would have expected higher levels, over the longer periods of time assessed.

Response
Thank you for the opportunity to explain the apparent low percentage of mCherry+ parasites. LHVS treated parasites recovered from transiently transfected CHO host cells are 10-15% mCherry+. Since only 20% of transiently transfected host cells are mCherry+ (FigureS5B), this means that 50-75% of the parasites exposed to mCherry have ingested mCherry. LHVS treated parasites recovered from inducible mCherry CHO cells are ~42% mCherry positive (FigureS5C). Because approximately 76% of inducible mCherry CHO cells are mCherry+, this indicates that roughly 55% of the parasites exposed to mCherry have ingested this reporter. Together this suggests that most parasites are actively endocytosing material from the host cytosol. It is possible that this is an underestimate for several reasons: (1) some of the transiently transfected mCherry+ host cells are dimmer than others; hence ingestion from these cells might not reach the threshold of detection within the parasite by microscopy; (2) although more uniform in expression, the inducible mCherry CHO cells are generally dimmer than the brightest transiently transfected cells (FigureS5), thus less active parasites might not accumulate sufficient mCherry for detection; (3) LHVS treatment inactivates CPL and probably CPB, but does not inactivate other yet-to-be-identified VAC proteases that are responsible for residual proteolytic activity in the VAC (Dou et al., mBio 2014). Accordingly ingested mCherry is still being turned over to some extent in LHVS treated parasites, further limiting detection in some parasites; and (4) although mCherry is more pH tolerant than e.g., GFP, the low pH of the VAC could compromise the signal in less parasites that are less trophic. To summarize, we do not know precisely what proportion of the parasites are actively endocytosing material from the host cytosol, but the available evidence suggests it is at least half of the population.

Additional changes to the revised manuscript:
Efforts were made to condense all sections of the manuscript. We also assessed the localization of ddGFP-DrpB WT within the T. gondii endolysosomal system (FigureS2) and colocalization of ingested host mCherry with ddGFP-DrpB DrpB (Figure 1) to compliment the data in Figure 7 addressing the role of DrpB in T. gondii endocytosis. Figures S3 and S4 were combined to accommodate a new Figure S2.

Decision and Reviews

Dear Dr. Carruthers, dear Vern,

Thank you for submitting your revised manuscript "Intersection of endocytic and exocytic systems in Toxoplasma gondii" to Traffic. I asked the referees to read the paper and their verbatim comments are appended below. I agree with the referees that you have adequately addressed the concerns raised previously and I am pleased to accept this paper for publication.

Sincerely,

Dominique Soldati-Favre, PhD
Associate Editor

Referee's Comments to the Authors

Referee: 2

Comments to the Author
I still find it a little disappointing that the authors have only included, what can only really be considered a negative result, as functional data. It can only be concluded here that DrpB is not involved directly in endocytosis. The authors say that more functional data is now being generated, but this will of course take much longer to analyse.

All other queries have been sufficiently addressed.

Referee: 1

Comments to the Author
In their revision, McGovern et al. addressed all my initial concerns. While the results regarding DrpB are not demonstrating a potential mechanisms for endocytic uptake and are somewhat confusing, the authors discuss these findings appropriately. It is out of the scope of this study to characterise additional, potential trafficking factors involved in endocytosis. As it stands, this would likely result in a huge fishing approach and would not be feasible within a realistic revision time.
Meanwhile, this study clearly demonstrates uptake via a "partially" conserved endocytic/exocytic transport system in Toxoplasma gondii and will be of general interest to the field.
I congratulate the authors to a nice and well performed study.