

An ER-resident lipid scramblase is crucial for biogenesis and function of apicomplexan parasite secretory organelles

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This paper entitled « An ER-resident lipid scramblase is crucial for biogenesis and function of apicomplexan parasite secretory organelles » by G. Srinivas Reddy et al describes the important role of the vacuole membrane protein 1 (VMP1), a DedA superfamily member of *T. gondii* (TgVMP1). They showed that PfVMP1 and TgVMP1 are ER-localized lipid scramblases. While none of their attempts to knock down PfVMP1 worked in *P. falciparum*, TgVMP1 depletion adversely affected parasite development, motility, host cell invasion, and egress. These phenotypes were linked to impaired rhoptry and dense granule biogenesis, and decreased secretion of micronemes in TgVMP1-depleted parasites, indicating a crucial role for TgVMP1. TgVMP1 depletion impaired lipid droplet homeostasis and ER organization. They rescued the ER-localized lipid scramblase by complementing TgVMP1-depleted parasites with PfVMP1 or a homolog as distant as human VMP1 rescued the depleted parasites, indicating their functional conservation and a crucial role for ER-resident lipid scramblase activity in the biogenesis and function of secretory organelles.

The manuscript is well written and the experiments were correctly designed and well-conducted. Therefore, I will recommend its publication after a minor revision. The data generated are almost convincing, except the comments below. The quality of this can be improved by answering the following concerns.

- 1) The authors wrote at the end of the abstract, the following sentence : « The essentiality of TgVMP1 for parasite development and likely functional conservation of apicomplexan VMP1 proteins highlight their drug-target potential ». This is unnecessary because it not convincing at all that PfVPM1 and TgVPM1, which are so conserved with the distant human counterpart and this later can perfectly complement the TgVMPM1 mutant, can be used for a drug-target design. Otherwise, the authors should tell how this can happen. I will suggest to remove this kind sentence, which have no true added value.
- 2) The authors do not discuss why all attempts in *P. falciparum* to knock down PfVPM1 do not work at all whereas there is no difficulty to genetically disrupt the function of TgVPM1 in *T. gondii*. What is the reason ? This is surprising since TgVPM1 and PfVPM1 are the same multilayer transmembrane proteins in these both closely related apicomplexan parasites.
- 3) They authors indicate several times that TgVPM1 is involved in the biogenesis of rhoptries and dense granules while it is not implicated in microneme biogenesis. The data provided to reach this conclusion are not convincing because only confocal microscopy and few antibodies have used to perform these experiments. An immuno-electron microscopy (IEM) using few antibodies specific to rhoptries, micronemes and dense granules should be used to see the subcellular distribution of gold-particles on these organelles. IEM will also demonstrate whether these proteins are properly located in the space destination after secretion.
- 4) The authors indicate that the secretion of ROP proteins are disturbed while ROP1 protein appears nicely and more concentrated in the PVM in the mutants relative to the parental parasites when carefully looking at the images provided. It will be more convincing to perform serial ultrathin sections in only one chosen small vacuole containing few intracellular parasites to obtain several images that will be superimposed to reconstitute the whole bodies of the mutants and parental parasites. These experiments will allow to determine whether they are less rhoptries in the mutants versus the wild type parasites because these organelles will be counted. The number of rhoptries in mutants vers wild type parasites may impact the level of protein secretion. In addition, this experimental strategy will show if the mutant parasites still have distal and basal micronemes because a negative impact in their distributions in the apical end of the parasites may also explain why there is an effect on their secretion.

Reviewer #2

(Remarks to the Author)

Here, the authors described in *Toxoplasma* and *Plasmodium* a lipid scramblase, the vacuole membrane protein 1 (VMP1), that they nicely showed to be associated with ER in both parasites. They present a range biochemical, ultrastructural and experimental cell biological data, to interrogate the role of *Toxoplasma* VMP1. They showed that parasites have a strong defect in replication, motility and invasion, that is associated with a defect in the biogenesis of their secretory organelles dense granules and rhoptries. Moreover, TgVMP1 depletion impaired lipid droplet homeostasis and ER organization, a phenotype that can be rescued with PfVMP1 or human VMP1.

The characterization of DedA-like protein in apicomplexa is novel. In other organisms, the cellular abnormalities observed due to the loss of VMP1 are multiple: fragmented ER, ER-contact, distorted Golgi, impaired endocytosis and exocytosis, damaged mitochondria, and accumulation of ubiquitinated-protein aggregates and lipid droplets (LDs)... Here the authors focused only on the biogenesis of secretory organelles and lipid droplets accumulation. The characterization of the secretory organelles in the mutant is incomplete. A full analysis is needed to better assess the importance of this DedA-predicted protein in parasites. Overall structural details of the mutant are missing and the interpretation of some results is confusing.

1. The authors performed TEM but the images shown do not allow drawing any conclusion because the poor preservation of the material : no intracellular membranes can be seen and therefore, ER and Golgi cannot be observed. This is particularly important for ER, since they claimed a change in the localisation of ER marker in the mutant (Figure S9). While they showed a strong defect in dense granules secretion, they do not analyze if dense granules are visible by TEM in the mutant ; they only focused on rhoptries. TEM should also resolves if micronemes are properly formed and present at the apex, including lateral micronemes, and sub-conoid micronemes. The analysis by IFA is not sufficient. Overall, the ultrastructure of the mutant by TEM is not conclusive and cannot support the claimed drawn by IFA, in particular, analysis of ER, dense granules, mitochondria.

2. The authors overlook the fact that the parasite has a very serious replication defect. 75% of vacuoles contains a single parasite. This can be explained by the absence of dense granule proteins secretion, which was recently shown to be important for intracellular parasite development (Chelaghma et al. *ELife* 2024 ; Fu et al. *PLoS Pathogen* 2023). However, the phenotype of replication is more drastic than in any of the mutants of annuli defective for secretion of dense granules (Chelaghma et al, *eLife* 2024 ; Fu et al., *PlosPathogen* 2023) or biogenesis of secretory organelles (Morlon-Guyot et al., *Cell Mic* 2015 ; Sloves et al. *Cell Host microbes*, 2012). This might suggest a role of lipid droplets homeostasis in growth, or potential other overlooked effects (point 1).

3. Since DedA is important in autophagosomes formation and that PI3K, ATG8, ATG3... are linked to Apicoplast in Apicomplexa, the presence and inheritance of the apicoplast during division need to be assessed.

4. The phenotype related to dense granules is paracellar. We would like to see further investigation of whether or not they are formed (TEM, see above). If they are visible, then to quantify secretion in more details (release in the media by quantification of GRAs in the ESA). The loss of IVM is suggested in TEM but the authors cannot quantify the association of GRA proteins with IVN by IFA (Fig 6B and S8B), we can just say that they are or not in vacuolar space. Association with IVN required specific fractionation procedures.

5. IFA figure 5A. The authors say that the rhoptries are fragmented. The resolution of IFA do not permit to see details of the morphologies of the rhoptries, even to see individual rhoptries. The ROP1 signal seems to be in the vacuolar space upon depletion of TgVMP1. First this should be confirmed by adding phase contrast. Second, the release of rhoptry proteins within the lumen is already observed upon depletion of proteins involved in rhoptry biogenesis (SORTILIN, VPS11, retromer complex...), and this is not discussed. Why there is still important signal in -IAA in panel 5A ?

6. GRASP-GFP staining is weird ; it normally looks like a bar above the nucleus near the ER. I don't think that they can use these images to claim colocalisation with Golgi.

7. The authors observed a strong defect in motility, secretion of micronemes and rhoptries. Since most of the parasites are not able to divide, it might be difficult to distinguish between a potential defect in parasite gliding motility vs. parasites that are dead or dying and thereby are unable to exhibit active motility. Same for microneme and rhoptry secretion assays.

8. Some of the results deserve more discussion.

-Any explanation of why dense granules proteins but not rhoptry proteins accumulated at the ER in the mutant ?

-VMP1 is an ER-resident protein so is expected to play a role in this compartment. Since the three secretory organelles, micronemes, rhoptry and dense granules, transit through the ER, why only dense granules and rhoptries are affected ? MICs proteins may follow, at some level, a different trafficking pathway ?

-it is surprising to see that 50% of mice survived after infection with the mutant (+IAA) while there is no plaques at all in vitro. The authors should comment on this discrepancy between in vitro and in vivo.

9. The interpretation of mass spectrometry data are questionable. The authors performed three independ IPs, but do not show any statistical significance of the enrichment, that will help for the conclusions, especially because almost all the proteins that they used to make their point are not enriched in the three sets of data. This is not clear how they connect an association of VMP1 with these proteins (MICs, GRAs, ROPs, IMC proteins) and biogenesis of secretory organelles.

10. The authors referred to the absence of network when they look at IFA of dense granule proteins. The IVN is not visible by IFA.

11. Fig. S4A. For the fusion of miniAID in Plasmodium, did the authors control by WB the expression of Tir1 in the transgenic line, which is a prerequisite for this conditional KD ?
12. Section PfVMP1 and TgVMP1 localize to ER. 'Towards characterization of apicomplexan VMP1 proteins, we replaced wild-type PfVMP1 and TgVMP1 coding sequences ...' This is not replacement of gene but endogenous tagging.
13. Figure S2 : Molecular sizes of PCR products on schematic and gels are missing.
14. Table : add accession number TGGT1

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have answered all questions raised by this reviewer. They provide additional data that are very convincing and the manuscript have been considerably improved. Their finding is of a high significance of the field. No other additional experiments are needed. The methodology sound very well and the conclusions are supported by the results obtained. Therefore, I will suggest to publish this work in Nature Communications.

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RESPONSES TO THE REVIEWER COMMENTS

Below are our responses to the reviewers' comments in a point-wise manner. The comments are *italicized* and excerpts from the manuscript are **highlighted** with the page No. and line No. corresponding to the start of excerpt in the file "Reddy, et al_Revised Manuscript".

REVIEWER #1

Comment 1. *The authors wrote at the end of the abstract, the following sentence: The essentiality of TgVMP1 for parasite development and likely functional conservation of apicomplexan VMP1 proteins highlight their drug-target potential. This is unnecessary.....I will suggest to remove this kind sentence.....*

Response. As suggested, we have removed the sentence "The essentiality of TgVMP1 for parasite development and likely functional conservation of apicomplexan VMP1 proteins highlight their drug-target potential" from the abstract on page 2.

Comment 2. *The authors do not discuss why all attempts in P. falciparum to knock down PfVPM1 do not work at all whereas there is no difficulty to genetically disrupt the function of TgVPM1 in T. gondii. What is the reason ? This is surprising since TgVPM1 and PfVPM1 are the same multilayer transmembrane proteins in these both closely related apicomplexan parasites.*

Response: We were also surprised with the failure of PfVMP1 knock-down in *P. falciparum*. We have discussed this point in the text on page 8, line 191: ".....**It is possible that intrinsic nature and ER membrane localization of PfVMP1 rendered it refractory to degradation in case of cDD and glmS systems, as has also been reported for other proteins^{44,45}. The auxin-inducible knock-down was quite effective for TgVMP1 in T. gondii but not for PfVMP1 in P. falciparum, which might be due to differences in the specificity of E3 ligases of P. falciparum and T. gondii for TIR1. Similar to our observation, previous studies showed limited success of the auxin-inducible system in P. falciparum⁴⁵.**"

Comment 3. *The authors indicate several times that TgVPM1 is involved in the biogenesis of rhoptries and dense granules while it is not implicated in microneme biogenesis. The data provided to reach this conclusion are not convincing because only confocal microscopy and*

few antibodies have used to perform these experiments. An immuno-electron microscopy (IEM) using few antibodies specific to rhoptries, micronemes and dense granules should be used to see the subcellular distribution of gold-particles on these organelles. IEM will also demonstrate whether these proteins are properly located in the space destination after secretion.

Response: We thank the reviewer for this valuable suggestion, and agree that confocal microscopy alone would not provide a complete picture of the secretory organelles. We have performed extensive TEM and IEM experiments to assess the effect of TgVMP1 depletion of micronemes, rhoptries, and dense granules. The TEM data are in agreement with the IFA data, and IEM data showed labelling of organelles with the corresponding marker protein-specific gold particles. The new figures and related results have been incorporated in the text as described below for each organelle:

Micronemes. Page 14, line 349: “Transmission electron microscopy (TEM) images confirmed the presence of both apical and lateral micronemes in +IAA and -IAA parasites (Supplementary Fig. 6C), which was also corroborated by MIC2 labelling in immunoelectron microscopy (IEM) images (Supplementary Fig. 7A). Furthermore, the TEM micrographs showed comparable number of apical micronemes in -IAA (9.9 ± 5.9 /apical region, $n=22$ parasites) and +IAA (9.0 ± 4.4 /apical region, $n=22$ parasites) parasites, which together with the IFA data indicate that TgVMP1 depletion did not affect microneme organization.”.

Rhoptries. Page 17, line 396: “.....For rhoptry organization, intracellular +IAA and -IAA tachyzoites were processed for IFA, TEM, and IEM. IFA images showed ROP1 localization to characteristic rhoptry-like elongated structures with a bulbous base in the majority (85.3%) of -IAA parasites as compared with only a small number (11.3%) of +IAA parasites (Fig. 5A, B). Similar to the intracellular parasites, purified -IAA parasites also exhibited ROP1 localization to rhoptry-like elongated structures, which was not the case with +IAA parasites (Fig. 5E). In agreement with the IFA data, TEM images showed apical rhoptries in 52.2% of -IAA parasites ($n=23$ parasites), whereas only 18.2% of +IAA parasites had apical rhoptries ($n=22$ parasites) (Fig. 5F). ROP1 labelling of the similar structures in IEM micrographs indicated ROP1 targeting to these structures (Supplementary Fig. 7B)”.

Dense granules. Page 20, line 483: “...TEM micrographs of both –IAA and +IAA parasites showed dense granules (Fig. 5F, 6C), which was corroborated by IEM showing GRA2 labelling of dense granules in the parasite cytoplasm and IVN in the PV (Supplementary Fig. 7C). The TEM micrographs of +IAA parasites contained less number of cytoplasmic dense granules (1.5/parasite section, n=153) than those of –IAA parasites (3.2/parasite section, n=94), which together with the IFA data indicates a crucial role of TgVMP1 in dense granule biogenesis. We next compared dense granule secretion in the ESA of purified –IAA and +IAA parasites by IEM using GRA2. The number of GRA2-gold particles was comparable (Supplementary Fig. 7D, E), suggesting that impaired dense granule biogenesis did not affect secretion. Nonetheless, majority of the gold particles were associated with electron dense material in –IAA parasites as opposed to +IAA parasites (Supplementary Fig. 7D), suggesting a defect in the post-secretion process that might be required for the formation of IVN.”.

Comment 4. *The authors indicate that the secretion of ROP proteins are disturbed while ROP1 protein appears nicely and more concentrated in the PVM in the mutants relative to the parental parasites when carefully looking at the images provided. It will be more convincing to perform serial ultrathin sections in only one chosen small vacuole containing few intracellular parasites to obtain several images that will be superimposed to reconstitute the whole bodies of the mutants and parental parasites. These experiments will allow to determine whether they are less rhoptries in the mutants versus the wild type parasites because these organelles will be counted. The number of rhoptries in mutants vers wild type parasites may impact the level of protein secretion. In addition, this experimental strategy will show if the mutant parasites still have distal and basal micronemes because a negative impact in their distributions in the apical end of the parasites may also explain why there is an effect on their secretion.*

Response: The reviewer’s comment is valuable, and we have addressed it below in a), b), and c).

a). *ROP1 protein appears nicely and more concentrated in the PVM in the mutants relative to the parental parasites when carefully looking at the images provided.*

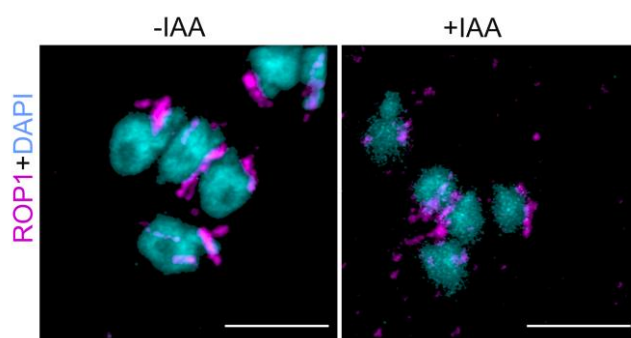
Response. Indeed, ROP1 signal appears to be predominant in the PV of +IAA parasites as compared to that of -IAA parasites, suggesting abnormal secretion. Nonetheless, in contrast to the –IAA parasite, ROP1 signal does not localize to characteristic rhoptry-like elongated structures in the +IAA parasites, indicating their absence. We have revised our analysis and

determined the number of +IAA and –IAA parasites with typical rhoptry-like elongated structures (Fig. 5A, B) or diffuse ROP1 signal in the PV (Fig. 5C, D). The results are described on page 17, line 397 (“...IFA images showed ROP1 localization to characteristic rhoptry-like elongated structures with a bulbous base in the majority (85.3%) of –IAA parasites as compared with only a small number (11.3%) of +IAA parasites (Fig. 5A, B). 59.4% of +IAA parasites also showed ROP1 signal in the PV compared to 10.9% of –IAA parasites (Fig. 5C, D)....”).

b). *It will be more convincing to perform serial ultrathin sections in only one chosen small vacuole containing few intracellular parasites to obtain several images that will be superimposed to reconstitute the whole bodies of the mutants and parental parasites. These experiments will allow to determine whether they are less rhoptries in the mutants versus the wild type parasites because these organelles will be counted. The number of rhoptries in mutants vers wild type parasites may impact the level of protein secretion.*

Response. We could not obtain serial ultrathin TEM sections corresponding to the same parasite to reconstruct a 3D image. Nonetheless, we carried out fresh TEM and IEM experiments, and added new data in support of the defects in secretory organelles in TgVMP1-depleted parasites, which is mentioned above in response to comment 3.

We also performed super-resolution Airyscan microscopy with 100 nm Z-slices, followed by 3D reconstruction of ROP1 labelling in –IAA and +IAA parasites. As evident from the below figure, the mutant parasites (+IAA) do not have typical elongated rhoptries.



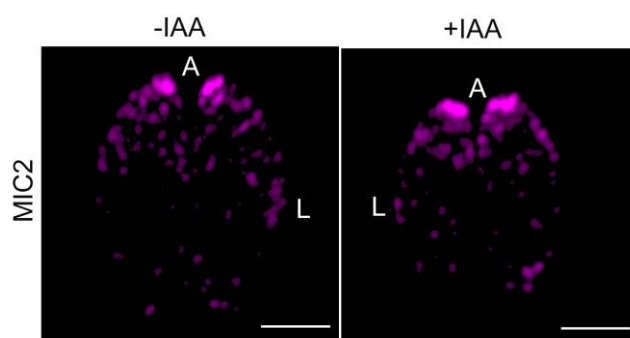
Accessory Fig. 1A. 3D reconstruction of ROP1 localization. –IAA and +IAA RHTIR1-VMP1AID parasites were evaluated for rhotpry organization by IFA using anti-ROP1 antibodies. Super-resolution Airyscan micrographs of the whole PVs were acquired using Zeiss LSM 880 confocal laser scanning microscope under the 100× objective with 100 nm optical

sections. The representative 3D reconstructed Airyscan image shows rhoptries (magenta) and nucleus DAPI (cyan) in a PV with 4 parasites. Note that ROP1 signal is restricted to characteristic rhoptry-like elongated structures in -IAA parasites, as compared with predominantly disordered structures in +IAA parasites. Scale bar = 5 μ m.

We could not count the number of rhoptries/parasite using the above 3D reconstruction images, as IFA does not offer the resolution required to distinguish individual rhoptries. Moreover, the number of rhoptries is variable in *Toxoplasma*, and it would also vary from section to section of the TEM images observed. Hence, we determined the number of parasites with apical rhoptries in mutant and control parasites, and it is included on page 17, line 402 (“.....**In agreement with the IFA data, TEM images showed apical rhoptries in 50% of -IAA parasites (n=24 parasites), whereas only 13.6% of +IAA parasites had apical rhoptries (n=22 parasites) (Fig. 5F). ROP1 labelling of the similar structures in IEM micrographs indicated ROP1 targeting to these structures (Supplementary Fig. 7B).....**”).

c). *In addition, this experimental strategy will show if the mutant parasites still have distal and basal micronemes because a negative impact in their distributions in the apical end of the parasites may also explain why there is an effect on their secretion.*

Response. We performed super-resolution Airyscan microscopy with 100 nm optical sections, followed by 3D reconstruction of MIC2 labelling in -IAA and +IAA parasites. The image shown below indicates that both the parasites contain apical and lateral micronemes.



Accessory Fig. 1B. 3D reconstruction of MIC2 labelling. -IAA and +IAA RHTIR1-VMP1AID parasites were evaluated for microneme organization by IFA using anti-MIC2 antibodies. Super-resolution Airyscan micrographs of the whole PVs were acquired using Zeiss LSM 880 confocal laser scanning microscope under the 100 $\times$ objective with 100 nm optical

sections. The representative 3D reconstructed Airyscan image shows localization of MIC2 in a PV with 2 parasites. Note MIC2 labelling in apical (A) and lateral (L) regions of both the +IAA and -IAA parasites, indicating the presence of apical and lateral micronemes. Scale bar = 2 μ m.

The above 3D reconstruction data is consistent with the IFA (Fig. 4A-D), TEM (Supplementary Fig. 6C), and IEM (Supplementary Fig. 7A) data, which show both apical and lateral micronemes in mutant and control parasites (page 14, line 347: “.....**All these proteins showed similar predominant apical localization in +IAA and –IAA parasites, ruling out any adverse effect of TgVMP1 in microneme organization (Fig. 4A-D; Supplementary Fig. 6A, B). Transmission electron microscopy (TEM) images confirmed the presence of both apical and lateral micronemes in +IAA and –IAA parasites (Supplementary Fig. 6C), which was also corroborated by MIC2 labelling in immunoelectron microscopy (IEM) images (Supplementary Fig. 7A). Furthermore, the TEM micrographs showed comparable number of apical micronemes in –IAA (9.9 ± 5.9 /apical region, n=22 parasites) and +IAA (9.0 ± 4.4 /apical region, n=22 parasites) parasites, which together with the IFA data indicate that TgVMP1 depletion did not affect microneme organization.**”).

We have not included the Accessory Fig. 1A and B in the text, as the included TEM micrographs offer better appreciation of rhoptries and micronemes in mutant vs control parasite. However, if desired by the reviewer, we will be glad to include these figures along with videos (uploaded on the portal) in the main text.

Reviewer #2

Comment 1. *The authors performed TEM but the images shown do not allow drawing any conclusion because the poor preservation of the material : no intracellular membranes can be seen and therefore, ER and Golgi cannot be observed. This is particularly important for ER, since they claimed a change in the localisation of ER marker in the mutant (Figure S9). While they showed a strong defect in dense granules secretion, they do not analyze if dense granules are visible by TEM in the mutant ; they only focused on rhoptries. TEM should also resolves if micronemes are properly formed and present at the apex, including lateral micronemes, and sub-conoid micronemes. The analysis by IFA is not sufficient. Overall, the ultrastructure of the mutant by TEM is not conclusive and cannot support the claimed drawn by IFA, in particular, analysis of ER, dense granules, mitochondria.*

Response: We greatly appreciate reviewer's suggestions. As suggested, we performed fresh experiments for TEM and IEM, and scanned several micrographs to evaluate the effects of TgVMP1 depletion quantitatively on secretory organelles. We have included brand new TEM and IEM micrographs and related results in the text as mentioned below.

Micronemes: Page 14, line 349: “Transmission electron microscopy (TEM) images confirmed the presence of both apical and lateral micronemes in +IAA and -IAA parasites (Supplementary Fig. 6C), which was also corroborated by MIC2 labelling in immunoelectron microscopy (IEM) images (Supplementary Fig. 7A). Furthermore, the TEM micrographs showed comparable number of apical micronemes in -IAA (9.9 ± 5.9 /apical region, $n=22$ parasites) and +IAA (9.0 ± 4.4 /apical region, $n=22$ parasites) parasites, which together with the IFA data indicate that TgVMP1 depletion did not affect microneme organization.”.

Rhoptries. Page 17, line 396: “.....For rhoptry organization, intracellular +IAA and -IAA tachyzoites were processed for IFA, TEM, and IEM. IFA images showed ROP1 localization to characteristic rhoptry-like elongated structures with a bulbous base in the majority (85.3%) of -IAA parasites as compared with only a small number (11.3%) of +IAA parasites (Fig. 5A, B). Similar to the intracellular parasites, purified -IAA parasites also exhibited ROP1 localization to rhoptry-like elongated structures, which was not the case with +IAA parasites (Fig. 5E). In agreement with the IFA data, TEM images showed apical rhoptries in 52.2% of -IAA parasites ($n=23$ parasites), whereas only 18.2% of +IAA

parasites had apical rhoptries (n=22 parasites) (Fig. 5F). ROP1 labelling of the similar structures in IEM micrographs indicated ROP1 targeting to these structures (Supplementary Fig. 7B)".

Dense granules. Page 20, line 483: "...TEM micrographs of both -IAA and +IAA parasites showed dense granules (Fig. 5F, 6C), which was corroborated by IEM showing GRA2 labelling of dense granules in the parasite cytoplasm and IVN in the PV (Supplementary Fig. 7C). The TEM micrographs of +IAA parasites contained less number of cytoplasmic dense granules (1.5/parasite section, n=153) than those of -IAA parasites (3.2/parasite section, n=94), which together with the IFA data indicates a crucial role of TgVMP1 in dense granule biogenesis."

LD. Page 22, line 536: "...TEM micrographs confirmed the presence of much larger size LDs in +IAA parasites than in -IAA parasites (Fig. 7D), indicating a key role for TgVMP1 in LD homeostasis. Additionally, LDs were closely associated with ER membrane in +IAA parasite, which was not evident in -IAA parasites."

ER. Page 24, line 585: "...ER-GFP was prominently clustered around the nucleus in a significant number of +IAA parasites (Fig. 7E, F). TEM micrographs showed ER tubules stretched throughout the +IAA parasites, including in close contact with LDs, whereas these were mostly perinuclear in -IAA parasites. These data indicate impaired ER organization..".

The method sections related to TEM and IEM have been revised as well (page 57-59).

Comment 2. *The authors overlook the fact that the parasite has a very serious replication defect. 75% of vacuoles contains a single parasite. This can be explained by the absence of dense granule proteins secretion, which was recently shown to be important for intracellular parasite development (Chelaghma et al. ELife 2024 ; Fu et al. PloS Pathogen 2023). However, the phenotype of replication is more drastic than in any of the mutants of annuli defective for secretion of dense granules (Chelaghma et al, eLife 2024 ; Fu et al., PlosPathogen 2023) or biogenesis of secretory organelles (Morlon-Guyot et al., Cell Mic 2015 ; Sloves et al. Cell Host microbes, 2012). This might suggest a role of lipid droplets homeostasis in growth, or potential other overlooked effects (point 1).*

Response: We thank the reviewer for bringing up this point to our notice. We agree that the replication defect upon TgVMP1 depletion is more severe than that has been reported in case of dense granule defect (Chelaghma, et al., 2024 (reference No. 66 in the text); Fu, et al., 2023 (reference No. 67 in the text)) or micronemes and rhoptries (Sloves, et al. 2012 (reference No. 56 in the text; Stasic, et al. 2029 (reference No. 57 in the text)). We acknowledge reviewer's point that this severe replication defect upon TgVMP1 depletion could be a combined result of defects in secretory organelles and LD homeostasis. We have discussed this point on page 32, line 808 (“...**The accumulation of enlarged LDs in close association with ER membrane in TgVMP1-depleted parasites indicates impaired LD homeostasis, which together with impaired secretory organelle biogenesis could have contributed to the overall block of parasite growth....**”).

The other potential reasons for severe parasite growth defect are discussed on page 32, line 790 (“...**Additionally, as IVN formation requires lipids from the host as a primary source and parasite as a secondary source^{9,65}, impaired lipid homeostasis in TgVMP1-depleted parasites could have limited lipid supply to the PV, thereby, adversely affecting IVN formation and parasite growth...**”), page 32, line 806 (“.....**ER-resident lipid scramblase activity is crucial for parasite development, which likely functions via translocation of lipids in the ER and the precursor vesicles destined to the secretory organelles.....This further indicates that the loss of ER-localized lipid scramblase activity is the major reason for impaired secretory organelle biogenesis, ER organization, LD homeostasis, and IVN formation in TgVMP1-depleted parasites, consequently blocking the parasite development.**”),

Comment 3. *Since DedA is important in autophagosomes formation and that PI3K, ATG8, ATG3... are linked to Apicoplast in Apicomplexa, the presence and inheritance of the apicoplast during division need to be assessed.*

Response: Indeed, human VMP1 has been shown to be required for autophagy during early stages (references 28 and 29). As suggested, we addressed this point by comparing -IAA and +IAA parasites for the presence of apicoplast using antibodies to the apicoplast marker protein ACP. We did not observe any noticeable difference in the presence of apicoplast across the number of -IAA and +IAA parasites observed, which has been mentioned on page 33, line 822: “....**HsVMP1 recruits the PI3K complex (VPS34/Beclin 1/Vps15) at the phagophore assembly site via interaction with Beclin 1, which is crucial for autophagosome formation^{28,29}. A striking consequence of the ablation of autophagy pathway in *T. gondii* and *P.***

falciparum has been shown to be the loss of apicoplast, a relict plastid that produces fatty acid precursors⁷⁰. We compared TgVMP1-depleted and control parasites for the presence of apicoplast as a marker to assess whether TgVMP1 depletion affected autophagy. The proportion of apicoplast-containing TgVMP1-depleted and control parasites was comparable (Supplementary Fig. 12), suggesting that the observed phenotypes of TgVMP1-depleted parasites are not associated with autophagy-related defects.”.

Comment 4. *The phenotype related to dense granules is parcelar. We would like to see further investigation of whether or not they are formed (TEM, see above). If they are visible, then to quantify secretion in more details (release in the media by quantification of GRAs in the ESA). The loss of IVM is suggested in TEM but the authors cannot quantify the association of GRA proteins with IVN by IFA (Fig 6B and S8B), we can just say that they are or not in vacuolar space. Association with IVN required specific fractionation procedures.*

Response: This is a valid point, and we thank the reviewer for prompting us to further address the effect of TgVMP1 depletion on dense granule biogenesis, secretion, and IVN formation. As has been described in response to comment 1, we have included brand new TEM and IEM data related to the effect of TgVMP1 depletion on dense granule biogenesis and IVN formation. The related excerpts from result and discussion sections are mentioned below.

Page 20, line 483: “...TEM micrographs of both –IAA and +IAA parasites showed dense granules (Fig. 5F, 6C), which was corroborated by IEM showing GRA2 labelling of dense granules in the parasite cytoplasm and IVN in the PV (Supplementary Fig. 7C). The TEM micrographs of +IAA parasites contained less number of cytoplasmic dense granules (1.5/parasite section, n=153) than those of –IAA parasites (3.2/parasite section, n=94), which together with the IFA data indicate a crucial role of TgVMP1 in dense granule biogenesis.. ...”.

We agree with the reviewer that IFA images showing diffuse GRA signal do not indicate loss of IVN. It was a misinterpretation on our part, and we have revised the concerned result section on page 19, line 467 (“...The number of parasites with diffuse GRA1 or GRA2 localization was significantly higher in +IAA parasites (83.6% for GRA1, 79.0% for GRA2) than that in –IAA parasites (9.6% for GRA1, 14.6% for GRA2) (Fig. 6B; Supplementary Fig. 8B). TEM images showed the absence of tubulo-vesicular IVN in the PV of 57.7% +IAA parasites (n=123 PVs) as compared with 7.8% –IAA parasites (n=64 PVs) (Fig. 6C), indicating a crucial role for TgVMP1 in IVN formation.”).

We quantified GRA2-gold particles as a measure of dense granule secretion in ESA by IEM. The results are mentioned on page 20, line 488 (“.....**We next compared dense granule secretion in the ESA of purified –IAA and +IAA parasites by IEM using GRA2. The number of GRA2-gold particles was comparable (Supplementary Fig. 7D, E), suggesting that impaired dense granule biogenesis in TgVMP1-depleted parasites did not affect secretion. Nonetheless, majority of the gold particles were associated with electron dense material in –IAA parasites as opposed to +IAA parasites (Supplementary Fig. 7D), suggesting a defect in the post-secretion process that might be required for the formation of IVN.**”).

Our IFA, TEM, and IEM data are consistent with each other, and show defects in dense granule biogenesis, post-secretion assembly of dense granule-secreted proteins, and IVN formation, which are discussed on page 31, line 775 (“.....**TgVMP1-depleted parasites contained less number of dense granules and also showed accumulation of dense granule proteins in the ER. The impaired ER organization in TgVMP1-depleted parasites might have affected the transport and assembly of dense granule proteins during transport through the ER/Golgi endo-membrane system, thereby, causing their accumulation in ER. Although fewer, the cytoplasmic dense granules in TgVMP1-depleted parasites showed normal secretion.....Both control and TgVMP1-depleted parasites secreted comparable GRA2 in the ESA. However, in contrast to TgVMP1-depleted parasites, majority of the GRA2 was associated with electron dense material in the ESA of control parasites.....This suggests a defect in the form of secreted-GRA2 or post-secretion assembly of GRA2 that might be necessary for IVN formation, ...as IVN formation requires lipids from the host as a primary source and parasite as a secondary source ^{9,67}, impaired lipid homeostasis in TgVMP1-depleted parasites could have limited lipid supply to the PV, thereby, adversely affecting IVN formation and parasite growth.**”).

Comment 5. IFA figure 5A. The authors say that the rhoptries are fragmented. The resolution of IFA do not permit to see details of the morphologies of the rhoptries, even to see individual rhoptries. The ROP1 signal seems to be in the vacuolar space upon depletion of TgVMP1. First this should be confirmed by adding phase contrast. Second, the release of rhoptry proteins within the lumen is already observed upon depletion of proteins involved in rhoptry biogenesis

(*SORTILIN*, *VPS11*, *retromer complex*...), and this is not discussed. Why there is still important signal in -IAA in panel 5A ?

Response: Once again, thank you for bringing up these valid points to our notice, which we inadvertently missed or misinterpreted. Certainly, IFA doesn't offer the resolution required to count individual rhoptries and assess rhoptry morphology. Accordingly, we have corrected the y-axis label of Fig. 5B, which now reads as: “%Parasites with normal rhoptries”. We have also rephrased the concerned sentence on page 17, line 400: “..**Similar to the intracellular parasites, purified -IAA parasites also exhibited ROP1 localization to rhoptry-like elongated structures, which was not the case with +IAA parasites (Fig. 5E).....**”).

We have added Fig. 5C with phase contrast to show ROP1 signal in the PV and determined the numbers of PVs with ROP1 signal (Fig. 5D), as mentioned on page 17, line 399 (“....**59.4% of +IAA parasites also showed ROP1 signal in the PV compared to 10.9% of -IAA parasites (Fig. 5C, D).....**”). We have discussed this result on page 31, line 766 (“...**the loss of apical rhoptries and secretion of ROP1 in the PV of TgVMP1-depleted parasites are similar to those observed in parasites with ablation of TgSORTLR, V-type proton ATPase or Vps11**^{56,57,65}. It is possible that TgVMP1 contributes to rhoptry biogenesis via interaction with TgSORTLR and V-type proton ATPase, and loss of this interaction in TgVMP1-depleted parasites could have adversely affected rhoptry biogenesis...”).

The faint signal in the “VMP1” panel of +IAA parasites in the previous Figure 5A could have been due to incomplete TgVMP1 depletion. However, it doesn't impact the result, as we have evaluated at least 100 PVs from each of the 3 independent experiments to quantitate the effect of TgVMP1 on rhoptries (Fig. 5B). We have replaced this panel in the revised manuscript with a better representative image.

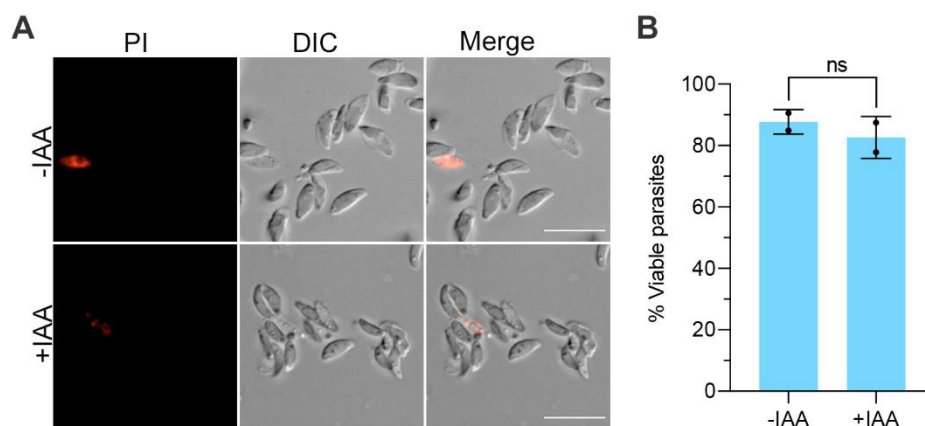
Comment 6. *GRASP-GFP staining is weird ; it normally looks like a bar above the nucleus near the ER. I don't think that they can use these images to claim colocalisation with Golgi.*

Response: We agree with the reviewer that GRASP-GFP localizes to a bar-like structure above the nucleus (*Bisio, et al., 2020; Palletier, et al. 2002*). We repeated the experiment to check whether TgVMP1 colocalizes GRASP-GFP. Indeed, we also observed that GRASP-GFP localization is predominantly bar-like close to the nucleus. We have revised Fig.1F, and mentioned the related result in the text on page 6, line 146 (“....**We episomally expressed ER-GFP or GRASP-GFP in RHTIR1-VMP1AID parasites, which showed substantial colocalization of ER-GFP with TgVMP1AID_{HA} (Fig. 1E, F), indicating that TgVMP1 is**

present in ER. On the other hand, only a minor proportion of TgVMP1 co-localized with GRASP-GFP in the region proximal to ER.....”).

Comment 7. *The authors observed a strong defect in motility, secretion of micronemes and rhoptries. Since most of the parasites are not able to divide, it might be difficult to distinguish between a potential defect in parasite gliding motility vs. parasites that are dead or dying and thereby are unable to exhibit active motility. Same for microneme and rhoptry secretion assays.*

Response: This is a valid and important comment. To address whether the observed defects could be due to parasite death, we set up the experiment exactly as been described in the method section “Effect of TgVMP1 depletion on gliding motility” on page 46, and performed propidium iodide (PI) staining of purified parasites as an indicator of cell death (Accessory Fig. 2A). As shown in the below Accessory Fig. 2B, the -IAA and +IAA parasites showed similar viability, indicating that the observed defects in motility, invasion, and organelle secretion are not due to the presence of dead parasites in the sample used in related experiments.



Accessory Fig. 2. Viability of parasites. RHTIR1-VMP1AID parasites were cultured with 0.1% ethanol (-IAA) or 500 μ M indole-3-acetic acid (+IAA) as has been described in the “Effect of TgVMP1 depletion on gliding motility” section. Purified -IAA and +IAA parasites were incubated with PI (5 μ g/ml in PBS) for 15 min at room temperature, washed with PBS, immediately observed under the 100 $\times$ objective of Zeiss Axioimager.Z2 fluorescence microscope, images were acquired using AxioVision software, and analysed using ImageJ software. **A.** The representative micrographs show PI staining (PI), DIC, and merge of the two panels (Merge). Scale bar = 10 μ m. PI staining indicates cell death. **B.** The number of PI-negative -IAA and +IAA parasites in “A” was counted and presented as a percentage of the total number of parasites observed (% Viable parasites) on y-axis for the indicated parasites on

x-axis. Each data is from 2 independent experiments (-IAA, n=324 cells; +IAA, 452 cells). The significance of difference between two data sets is indicated (ns: not significant).

We have not included this figure in the text, as there are already 8 main figures and 13 supplementary figures. Nonetheless, we have included the result on [page 13, line 283](#) (“.....the viability of purified +IAA (82.6% ± 6.8) and -IAA (87.7% ± 4.0) RHTIR1-VMP1AID tachyzoites was comparable, indicating that the decreased motility of +IAA parasites is not associated with parasite viability.”).

Furthermore, the purified -IAA and +IAA parasites used for conoid extrusion, which were obtained using the similar procedure, did not show any significant difference in conoid extrusion (Supplementary Fig. 5). This data also ruled out differences in viability of purified -IAA and +IAA parasites.

Comment 8. *Some of the results deserve more discussion.*

-Any explanation of why dense granules proteins but not rhoptry proteins accumulated at the ER in the mutant ?

-VMP1 is an ER-resident protein so is expected to play a role in this compartment. Since the three secretory organelles, micronemes, rhoptry and dense granules, transit through the ER, why only dense granules and rhoptries are affected ? MICs proteins may follow, at some level, a different trafficking pathway ?

-it is surprising to see that 50% of mice survived after infection with the mutant (+IAA) while there is no plaques at all in vitro. The authors should comment on this discrepancy between in vitro and in vivo.

Response: We agree with the reviewer's points. This comments has 2 points, as suggested by the reviewer, we have responded to these points below in a) and b).

a). *“Any explanation of why dense granules proteins but not rhoptry proteins accumulated at the ER.....MICs proteins may follow, at some level, a different trafficking pathway ?”.*

Response. We have discussed this point on [page 32, line 794](#): **“Different effects of TgVMP1 depletion on micronemes, rhoptries, and dense granules, could be due to differences in the pathways leading to their biogenesis and secretion^{3,4,6,49,67,68}. It is possible that TgVMP1 directly/indirectly regulates the proteins in ER/Golgi that are involved at different stages of transport/maturation of the proteins destined to these organelles,**

which is also supported by the presence of several proteins in TgVMP1 interactome that regulate biogenesis of secretory organelles at different stages. Similar observations have been reported earlier for TgSORTLR and V-type proton ATPase, which are required for trafficking of microneme and rhoptry proteins^{56,57}, but not for dense granule proteins. Similarly, depletion of apical annuli-associated proteins have been shown to have different effects on dense granule secretion and plasma membrane proteins.....”.

b). it is surprising to see that 50% of mice survived after infection with the mutant (+IAA) while there is no plaques at all in vitro. The authors should comment on this discrepancy between in vitro and in vivo.

Response. We have discussed the difference in the effects of TgVMP1 depletion on in vitro and in vivo parasite viability on page 9, line 224 (“This is in contrast to the complete block of parasite development upon TgVMP1 depletion in plaque assay, which might be due to differences in auxin bioavailability in in vitro and in vivo experimental settings.”).

Comment 9. The interpretation of mass spectrometry data are questionable. The authors performed three independent IPs, but do not show any statistical significance of the enrichment, that will help for the conclusions, especially because almost all the proteins that they used to make their point are not enriched in the three sets of data. This is not clear how they connect an association of VMP1 with these proteins (MICs, GRAs, ROPs, IMC proteins) and biogenesis of secretory organelles.

Response: We thank the reviewer for bringing up this point to our notice. We reanalysed our mass spectrometry data for statistically significant peptide enrichment in the TgVMP1AID_{HA} immunoprecipitate compared to the control as mentioned in the Method section on page 53, line 1463 (“....The peptide abundances in RHTIR1 and TgVMP1AID_{HA} immunoprecipitates from 3 biological repeats were compared for enrichment analysis using MetaboAnalyst 6.0 (<https://www.metaboanalyst.ca/>) with a single factor statistical analysis. Proteins enriched ≥ 1.5 -fold with a *P*-value < 0.05 in TgVMP1AID_{HA} immunoprecipitate compared to the RH-TIR1 immunoprecipitate were considered significant interactors of TgVMP1.”).

The revised supplementary Table 2 only contains proteins that are ≥ 1.5 -fold enriched with *p*-value ≤ 0.05 in TgVMP1 immunoprecipitate. The revised data contains multiple proteins associated with micronemes, rhoptries, and dense granules (page 25, line 597: “....TgVMP1 interactome contained proteins that have been shown to localize to micronemes (apical

cap protein AC5 and MIC7), rhoptries (RON3, ROP8, ROP26, and ROP37), and dense granules (phosphatidylserine decarboxylase, cathepsin CPC2, and GRA12).....gliding motility (PhiL1, GAPM3, GAPM2B, GAPM1A, and GRA12), processing of the microneme and rhoptry proteins (V-type proton ATPase subunit a1), and vesicle transport (sortilin-like receptor, Rab5b, glutathione S-transferase 2, and Rab2).....”).

Since the revised data also contains significantly enriched multiple organelle or organelle-associated proteins, it is logical to propose that TgVMP1 may contribute to the functioning and biogenesis of the secretory organelles via these proteins.

Comment 10. *The authors referred to the absence of network when they look at IFA of dense granule proteins. The IVN is not visible by IFA.*

Response: We agree with the reviewer, and apologise for misinterpretation of the IFA data (Fig. 6B, Supplementary Figure 8B). Indeed, IFA images showing diffuse GRA signal does not indicate loss of IVN. We have revised the concerned result section and determined the number of –IAA and +IAA PVs with IVN based on TEM micrographs on page 19, line 467 (“....The number of parasites with diffuse GRA1 or GRA2 localization was significantly higher in +IAA parasites (83.6% for GRA1, 79.0% for GRA2) than that in –IAA parasites (9.6% for GRA1, 14.6% for GRA2) (Fig. 6B; Supplementary Fig. 8B). TEM images showed the absence of tubulo-vesicular IVN in the PV of 57.7% +IAA parasites (n=123 PVs) as compared with 7.8% –IAA parasites (n=64 PVs) (Fig. 6C), indicating a crucial role for TgVMP1 in IVN formation.”).

Comment 11. *Fig. S4A. For the fusion of miniAID in Plasmodium, did the authors control by WB the expression of Tir1 in the transgenic line, which is a prerequisite for this conditional KD ?*

Response: This is a valid point. As suggested, we performed western blot of PfVMP1AID parasites to check TIR1 expression using anti-TIR1 antibodies. We have discussed this point in the text (page 8, line 189: “..... PfVMP1AID parasites expressed TIR1 (Supplementary Fig. 3I), which ruled out failure of auxin-inducible knock-down due to its absence.”).

Comment 12. *Section PfVMP1 and TgVMP1 localize to ER. ‘Towards characterization of*

apicomplexan VMP1 proteins, we replaced wild-type PfVMP1 and TgVMP1 coding sequences ...' This is not replacement of gene but endogenous tagging.

Response: We have rephrased this and other similar sentences on page 6, line 132 (“...**the endogenous** PfVMP1 and TgVMP1 coding sequences in *P. falciparum* D10 and *T. gondii* RHTIR1 strains **were tagged in-frame at their 3'-ends** with the coding sequences of GFP and AID_{HA}, respectively.”), page 8, line 178 (“...**the endogenous PfVMP1 coding region was tagged in-frame at the 3'-end with cDDHA-glmS ribozyme coding sequence in *P. falciparum* D10 strain to obtain a trimethoprim- and glucosamine-inducible conditional knock-down line (PfVMP1dKD)...**”), and page 8, line 182 (“..**the endogenous PfVMP1 coding region was tagged in-frame with mAID_{HA}-PcDT5U/TIR1-Flag-T2A/HD/PcDT3'U sequence in *P. falciparum* 3D7 strain to generate an auxin-inducible knock-down line (PfVMP1AID)...**”).

Comment 13. *Figure S2 : Molecular sizes of PCR products on schematic and gels are missing.*

Response: We thank the reviewer for such a detailed review and bringing up this point to our notice. We have included molecular size markers and the sizes of expected PCR products in Supplementary Fig. 3 and 4.

Comment 14. *Table : add accession number TGGT1*

Response: As suggested, we have included both Uniprot and ToxoDB IDs for each protein in Supplementary Table 2.

References

Bisio, et al. The ZIP code of vesicle trafficking in Apicomplexa: SEC1/Munc18 and SNARE proteins. *MBio*. 2020, 11(5), 10-1128.

Pelletier, L. *et al.* Golgi biogenesis in *Toxoplasma gondii*. *Nature*. 2002, **418**, 548-552.