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Plasmodium sporozoite disintegration during skin passagelimits malaria parasite transmission

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Review #1

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

The manuscript by Kehrer et al present a very nicely conducted and interesting findings on a protein concavin that is responsible for the shape and structure of sporozoite stage of malaria parasite. They also used Toxoplasma to study the function of gene. In malaria parasite using rodent malaria model P berghei, they showed its location and function on the sporozoite stage of parasite. Their experiment reveal that gene deletion of concavin affect the contour of the sporozoite specially during its invasion in salivary gland of the mosquito host and also during the passage with in the skin of the mammalian host. They performed very pertinent and well controlled experiments to substantiate these finding. It is pleasure to see that not only main figures but the supplementary figures are so well presented and explained. The literature and prior studies was well covered. They also show that this protein is part of the membrane associated protein that is facing cytoplasm but is necessary to maintain the shape. It was a pleasure to review this manuscript.
My few comments and some experiments which may be the authors have already performed but did not include are following manuscript. I am not suggesting that these all to be done as I am aware of covid situation. But if the authors can discuss or add few like at gametocyte or ookinete stages then it will strengthen the manuscript.

1. I understand that authors studied this protein as they identified this protein as putatively secreted protein in their mass spectrometry line 112-115 (Kehrer et al 2016). So I am not sure if this protein is indeed secreted and if it then how do the authors explain its location in the membrane? Is it involved in some secretory vesicle or was it a contamination with surface protein? Did they try to do the mass spec using this protein? Can they show by subcellular western blot analysis if this surface protein or integral membrane or plasma membrane associated protein?
2. The authors do say that they find the protein in the gametocyte but it is not clear whether it was in activated gametocyte male/female), zygote or present in inactivated gametocyte. They compared this protein with Phil1 but as shown earlier that Phil1 was very much a polarity protein expressed in female activated gametocyte. So is this protein shows some polarity at earlier gametocyte stages or during ookinete stages or during sporozoite maturation about which author do not discuss much. May be this change is not only due to shape but the result of the polarity markers being not expressed.
3. The authors touched a bit on the protein like GAP45 in the discussion but do they have any information on

the IMC proteins as well as some of the membrane protein like GAPMs which span the membrane. Did they try to look if the expression of these protein being affected. Did they perform the protein pulldown with their concaved GFP to look at the other proteins part of this complex on the membrane.
4. Its is very interesting to note that concavin deleted parasite lose their shape during migration into salivary duct. What is the phenotype in haemolymph? Authors do not mention about it. Does the maturation of sporozoites within oocyst affect the shape as well? Is there anything affected with egress of the sporozoite?
Minor comments
1. May be the nomenclature of Pf concavin (Fig. 2) can be changed as it is misleading to say Pf concavin since the protein of Pf concavin is studied in chimera of Plasmodium berghei expressing rather than the gene deletion in P.falciparum.
2. Can author please mark the apical end of the parasite?

Overall a very nice work and really enjoyed reviewing it.

3. Significance:

Significance (Required)

The work is more focussed on the gene involved in the maintenance of cell shape malaria parasite but may have interest to other cell biologist as well. The various way and assay the authors have used can be useful for community studying cell invasion.

My expertise is on the cell biology of malaria transmission stages.

Review #2

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

In this study, the authors characterize the function and localization of a Plasmodium sporozoite related protein which they name concavin due to its impact on the characteristic sporozoite shape. In summary, the

protein was identified by proteomics analysis of secretion (in combination with prev. studies) and P. berghei concavin knockouts (concavin(-)) were generated to study function in multiple Pb life stages in vitro and in vivo. Fluorescent-tagged Plasmodium lines were generated to localize concavin (in comparison to PHIL1, a subpellicular network related protein; concavin-GFP and PHIL1-GFP)) and assess subcellular mobility (e.g. FRAP). The study is well executed, controlled and written and the findings suggest that concavin is a mobile protein that plays an important role in mammalian infection by maintaining the sporozoites' shape when it enters the narrow salivary glands and proboscis in mosquitoes and during migration through the skin en route to the liver.

Major comments

The authors provide a comprehensive and detailed study which is well structured. However, they do not consider the already existing literature of this Plasmodial protein and its effect on mosquito-stage P. berghei parasites. Previously, this protein was studied by Ukegbu et. al 2021 Frontiers in Cellular and Infection Microbiology, in which the authors performed a screen and knockout studies of PBANKA_1422900 in P. berghei, which they call PIMMS22 (also the name in PlasmoDB). Ukegbu and colleagues investigated transmission to the mosquito and showed a modest defect in oocyst production, even when mutant ookinetes were injected into the midgut epithelium, demonstrating some role in ookinete-to-oocyst development at the basal lamina (rather than midgut traversal). Ukegbu et al performed localization studies including in sporozoites but did not report a defect in sporozoite shape. However bite back experiments failed to cause patent infection in mice, demonstrating PIMMS22 is also required to infect the mammalian host. The current manuscript by Kehrer et al nicely adds information by providing further evidence of why mammalian infection is blocked - a subset of KO sporozoites have abnormal shape that precludes their normal movement in the mosquito, including through the proboscis, and in the mammalian skin. The authors should integrate the earlier study into the manuscript including:

- correcting their statement of identifying a new protein
- mention their differing phenotypes regarding oocysts and list any experimental parameters that can be identified to differ between studies that could account for the oocyst differences
- consider the gene nomenclature, perhaps harmonising the name concavin with PIMMS22.

With regard to the concavin(-) phenotype, it is notable that only a subset of sporozoites have the shape defect, which changes over time and parasite location. The reasons for this should be further explained (e.g. could it be heterogeneity of population, sporozoite age, location, maturity etc?).

In addition, I have a few suggestions which would improve the study.

1. The authors claim in line 395 and throughout the manuscript that concavin(-) sporozoites reveal for the first time a loss of cellular integrity as phenotypic consequence. However, this is only the case for 20% of concavin(-) sporozoites in salivary glands on day 17, for 70% on day 28 (Figure 1D) and for 30-40% of concavin (-) sporozoites in the skin (line 306). It would be important to discuss why this occurs only in some and not in all concavin(-) sporozoites which were able to enter narrow spaces (SG and skin)?
2. Somewhat related to comment 1, the authors performed extensive experiments to describe the phenotype of concavin(-) parasites and demonstrate the physiological effect. However, how concavin acts is still unclear.
3. P. berghei is a powerful model and some analysis of the orthologous gene in human infecting Plasmodium species would further strengthen the significance of this study in relation to malaria being a disease of humans.
4. Concavin is essential for efficient transmission of mosquitoes, but anywhere else? The authors demonstrated a delayed prepatency period for concavin(-) sporozoites via bites of mosquitoes, demonstrating a strong defect somewhere between injection of sporozoites and the blood stage. Is it all at the mosquito and

skin? To validate their hypothesis that this delay is specifically due to inefficient ejection and migration of sporozoites through the skin, the authors should perform an experiment with dissected sporozoites and inject them intravenously. This would uncouple mosquito feeding to the remaining steps (ie answer whether there is also a liver stage phenotype?).

Minor comments

5. Significance Section. It would be beneficial for the reader to add a line or two discussing the significance of the finding in regards to application or translation.
6. Introduction. The authors mention IMC-proteins (line 85), secretion/pore forming secretion proteins (line 90 and 98). It would be beneficial to define those (e.g. in brackets).
7. Introduction. The authors mention the protein LIMP (line 103). An explanation of this abbreviation would add more clarity.
8. Figures. As outlined below in more detail, for some figures and graphs the number of independent repeats per experiment is not clearly described in figure legends. The author should include more detailed information about the number of independent experiments and/or samples used to demonstrate reproducibility of results.
a. Figure 1B. 32 individual Oocysts counted. Were additional independent transmission experiments performed and if so how many? Number of repeats has to be mentioned and n=3 is standard.
b. Figure 1C; Sporozoite motility assay. No information about number of repeats
c. Figure 1E. Number (n) of mosquitoes is not reported.
d. Figure 1F. How many independent experiments per group? Why are the round sporozoites not included in the graph and at which timepoint was it measured?
e. Figure 3. Number of independent repeats for each experiment of this figure needs to be stated
f. Figure 4. Independent repeats need to be reported
g. Figure 6D. Independent experiments need to be reported
9. Figure 4A. The figure includes two scans with different 2D dimensions for wildtype sporozoites (lengthwise vs 'cut through') but only one image/dimension for the mutant sporozoites. The authors might explain why or include a scan of a different sporozoite orientation to prove the round shape.
10. Figure 5F. Form the figure it is not exactly clear which parasites are normal and deformed. The authors should indicate deformation with arrows, for example.
11. Figure 6C. There is no figure legend for the graph in the middle with describes cells [%] on the y-axis. It is assumed, it shows the percentage of deformed and normal SPZ of intracellular concavin(-) sporozoites, however, this is not 100% clear. The authors should make this clearer.
12. Figure 7A and B. The authors should add statistical analysis here as in other figures.
13. Figure S1. Expression level of concavin in Ookinetes suggest potential importance in that life stage? It would be interesting to discuss this, particular in context with existing literature, see comment below.
14. Figure S3. I believe the meaning of concavin(-) NS abbrev. is not explained at any part of the manuscript. Please declare.
15. Figure S8 legend. Scale bar declaration is incomplete. Please correct or delete as it is part of figure itself already.

3. Significance:

Significance (Required)

A well executed and communicated study that will be of interest to malaria researchers and likely also other cell biologists. The authors should discuss their results in context with the existing literature and carefully navigate any differences to earlier work. Overall, the findings of this study add very nicely to existing

literature on the function of this protein and have impact on further studies of the motility and cell shape of Plasmodium sporozoites in different migratory processes.

My expertise is in malaria genetics and cell biology including transmission and liver stages.

Review #3

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

This work provides a forensic characterisation of the role of a palmitoylated protein of unknown function, essential for maintaining the svelte cell shape in malaria sporozoites. Initially using a classic experimental genetic approach the authors investigate its role and localisation using knockout and tagging, revealing it to localise to the sporozoite periphery, between plasmamembrane and inner membrane complex. They also reveal that gene knockout results in a morphological degeneration phenotype in salivary gland sporozoites. The work is set apart by the exceptional analysis of this phenotype in both in vitro, ex vivo and in vivo experiments to determine where they are able to meticulously demonstrate the link between structural integrity, motility, and key steps in the transmission process. The work has a clear narrative and applies an array of cutting-edge imaging approaches in a challenging mosquito/parasite system. Conclusions are well supported and data and statistical analysis are robust throughout.

I just have a couple of major points which can be addressed with changes to text, as well as a number of minor points for the authors to consider.

Major comments:

Ookinete phenotype? Whilst concavelin localises to both ookinete and sporozoite peripheries, and hints of a pre-oocyst phenotype there is almost no mention of ookinetes in stark contrast to the forensic examination in sporozoites. Could the authors comment on whether any deformation was seen in ookinetes and if so whether the deformation changed over time within cultures? Were any experiments carried out on ookinete motility for example? New experiments would not be necessary but some expanded discussion in this area is

important.

Previous gene description - A paper earlier this year has been published describing a phenotype for this gene Identification of Three Novel Plasmodium Factors Involved in Ookinete to Oocyst Developmental Transition (nih.gov) . Notably there is almost no overlap in the analysis other than defining the gene as important for transmission. Indeed the work focuses predominantly on the preoocyst phenotype so is extremely complementary. The gene has a screening name of PIMMS22, but I don't think precludes the use of a new name now its functional role has been delineated. Consider mentioning the paper briefly in the intro and incorporate fully into the discussion. If no data is available to address the ookinete questions above, then referencing here may be useful for that too.

Minor comments:

Introduction - excellent background to sporozoite biology, but add a few sentences to note that key structures and processes are conserved across other malaria zooites.

Line 109- 114 The background here is a little confusing as it starts by highlighting importance of identifying surface proteins and the fact concavin came out of a screen for secreted proteins. It ends up clearly not being surface or secreted protein, so I would simplify this section just highlighting that it came out of a sporozoite proteomics screen and perhaps other reasons it looked interesting.
Line 115 - clarify the delineation of previous and current work. Perhaps something "Here we..."
131 "expressed highest" doesn't sound quite right, should be changed to something like "to expressed at highest abundance" or "expression peaks in..."
Fig S2 legend states palmitoylation site is C terminus, but alignment suggests N. Also L136 says N, and L376 says C. Should they all be N?

Figure 1E seems redundant and displays same data as D (which I prefer).
Figure 1F shows data for deformed but not round sporozoites. Do they exhibit any forward motility? Do round sporozoites display any processing on the spot, or spinning (perhaps a video if so?)? This would indicate they remain alive but uninfectious purely down to inability to move forward.

Fig 2c add a note on the inset salivary gland images in the legend (not mentioned). Also add scale bar to the insets. There is signal in both (and stronger signal in the Pb comp?) but its not completely obvious what the message is without any mention in legend. Insets should be bigger too (tighter crop?).

L269 - Clarify this sentence. Could you also not confidently see deformed sporozoites or just rounded? Were there 10-20% unclassifiable and therefore possible deformed?

Morphology of nucleus - Could the authors state whether the deformation of the nucleus is altered (from it notably thin morphology seen in sporozoites) and whether it is associated with deformation and break points? In other stages constriction of nucleus is important for navigating small spaces. Perhaps some discussion of this work is relevent https://doi.org/10.15252/embr.201948896

L375 and following. I am not sure I follow the logic of it being more likely to be linked to PM? Whilst we know many IMC proteins are anchored to the subpellicular network, and thus immobile, would one associated with IMC by palmitoylation be expected to be?
Importantly, the palmitoylation mutant experiment demonstrates that it is not required for the peripheral localisation. This should be discussed, and strongly suggests that it binds to an IMC or PM protein. As an aside note, identification of complexes would be a really exciting next step in future work, and pulldowns

might be best carried out using in vitro cultured ookinetes or tachyzoites?

Was an attempt made to tag at N terminus? Although it would have disrupted the Palmitoylation site, it may have been interesting to compare the N-terminal vs C terminal location with STED, to test the IMC spanning hypothesis.

L400 and following. I am also strongly reminded of the degenerative ookinete phenotype observed in PDEd KO P. berghei. These parasites also undergo a progressive change from slender ookinetes to deformed and then round. There are some interesting parallels in the IMC disruption, and impeded motility (with intact motor) with rounded forms processing on the spot https://doi.org/10.1371/journal.ppat.1000599 Could be worth discussion.

L423, again unless the authors explain the link with secretion, it may be clearer to leave out the statement regarding its identification.

3. Significance:

Significance (Required)

The work presents an important conceptual advance in both the key role of a malaria protein of previously unknown function as well as a deeper understanding of the relationship between cell shape, motility and interactions with distinct host environments. Apicomplexan parasites are highly polarised and their morphology is underpinned by unique structures including the inner membrane complex and an associated actomyosin motor. Whilst several protein families are known to contribute to these structures, concavin represents a new class conserved in all apicomplexans. Its loss seems to be particularly disruptive in sporozoites, and the detailed analysis demonstrates both that this structural integrity is vital for supporting cell migration - and critically this is most important at the key boundaries of salivary gland secretion and skin migration of sporozoites.

The combination of technical approaches taken to examine the phenotype in sporozoites, is also exemplary and will be of significant interest to those working on difficult to image vector-borne pathogens.

The work will be of great interest to those studying host pathogen interactions, cellular motility systems as well as those working on invasive stages of malaria parasites.

A previous knockout of this gene has been published but did not identify the key morphological sporozoite phenotypes nor the specific barriers to transmission encountered. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8005625/
As such I do not feel this has any negative impact on significance of this particular work.

My field of expertise is in molecular parasitology, particularly in experimental genetics of malaria parasites, as well as motility and invasion of malaria parasites.

We would like to thank the reviewers for their overall very positive comments and constructive suggestions on our work that helped us improve our manuscript and provided interesting input for our future research. We particularly appreciate the notion of the reviewers who think our work is of interest not just to infection disease researchers but to a broader cell biology community. Reviewers 1 and 2 clearly note the interest to a broad cell biology community with reviewer 2 also recognizing the translational potential and reviewer 3 notes an interest for researchers working on host pathogen interaction and cellular motility systems.

In order to address the comments we performed new experiments: (i) infecting A. gambiae mosquitoes (added to Figure S4), (ii) repeating infections of *concavin(-)* parasites (added to Figures 2 and 6), the point mutant and the *concavin(-)* complementations (added to Figure 5), (iii) in vitro ookinete cultures (added to Figure 1) and (iiii) intra venous injections of sporozoites (added to Figure 6).

We hope that the additional work and the adapted text are now making this a paper of interest to a broad community of scientists interested in both basic and translational science.

Please see below our point-to-point response (in blue) to your queries. Changes in the manuscript itself are highlighted with track changes.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

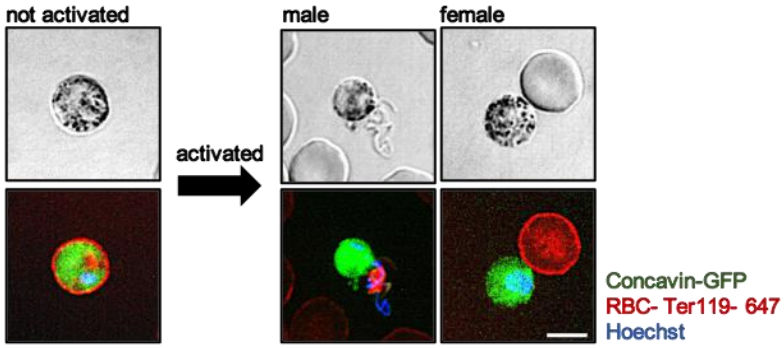
The manuscript by Kehrer et al present a very nicely conducted and interesting findings on a protein concavin that is responsible for the shape and structure of sporozoite stage of malaria parasite. They also used Toxoplasma to study the function of gene. In malaria parasite using rodent malaria model P berghei, they showed its location and function on the sporozoite stage of parasite. Their experiment reveal that gene deletion of concavin affect the contour of the sporozoite specially during its invasion in salivary gland of the mosquito host and also during the passage with in the skin of the mammalian host. They performed very pertinent and well controlled experiments to substantiate these finding. It is pleasure to see that not only main figures but the supplementary figures are so well presented and explained. The literature and prior studies was well covered. They also show that this protein is part of the membrane associated protein that is facing cytoplasm but is necessary to maintain the shape. It was a pleasure to review this manuscript.
My few comments and some experiments which may be the authors have already performed but did not include are following manuscript. I am not suggesting that these all to be done as I am aware of covid situation. But if the authors can discuss or add few like at gametocyte or ookinete stages then it will strengthen the manuscript.

1. *I understand that authors studied this protein as they identified this protein as putatively secreted protein in their mass spectrometry line 112-115 (Kehrer et al 2016). So I am not sure if this protein is indeed secreted and if it then how do the authors explain its location in the membrane? Is it involved in some secretory vesicle or was it a contamination with surface protein? Did they try to do the mass spec using this protein? Can they show by subcellular western blot analysis if this surface protein or integral membrane or plasma membrane associated protein?*

Response: At the current stage we assume the protein is not secreted to the outside of the parasite (see Figure 2F and 3C) but it might well be part of secretory vesicles and deposited in the plasma membrane. Like for GFP-tagged CSP (Singer and Frischknecht 2021), which must be trafficked and secreted somehow, we don't see it in secretory vesicles. We have now decided to rephrase this part to not confuse readers and directly focus on the characterization of the protein. Doing pulldowns or proximity labelling to find neighboring proteins hinting to a possible function is planned as a follow-up study. Pending on the nature of the interacting proteins, we might then also include subcellular western blots in a subsequent study.

2. *The authors do say that they find the protein in the gametocyte but it is not clear whether it was in activated gametocyte male/female), zygote or present in inactivated gametocyte. They compared this protein with Phil1 but as shown earlier that Phil1 was very much a polarity protein expressed in female activated gametocyte. So is this protein shows some polarity at earlier gametocyte stages or during ookinete stages or during sporozoite maturation about which author do not discuss much. May be this change is not only due to shape but the result of the polarity markers being not expressed.*

Images shown in Figure 2 represent non- activated gametocytes. We now made this clear in the text and have also included more detailed images of activated vs non-activated gametocytes as Figure S7 using the RBC marker Ter119 as counter staining. We did not observe a specific protein localization / polarization upon activation as shown here for convenience:



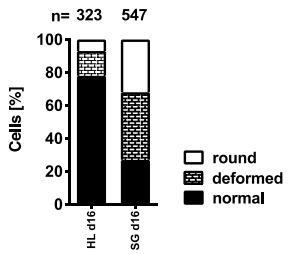
Phil1-GFP in activated concavin(-) gametocytes and fully developed ookinetes localizes similar to Phil1- GFP in wild type parasites (See Figure S7). We thus conclude that cell polarity appears normal in parasites lacking concavin.

3. *The authors touched a bit on the protein like GAP45 in the discussion but do they have any information on the IMC proteins as well as some of the membrane protein like GAPMs which span the membrane. Did they try to look if the expression of these protein being affected. Did they perform the protein pulldown with their concaved GFP to look at the other proteins part of this complex on the membrane.*

Thanks for suggesting these experiments, which will eventually help us to understand concavin function. As mentioned above, pulldown and probing interactions with these proteins is planned as part of a follow up study.

4. *Its is very interesting to note that concavin deleted parasite lose their shape during migration into salivary duct. What is the phenotype in haemolymph? Authors do not mention about it. Does the maturation of sporozoites within oocyst affect the shape as well? Is there anything affected with egress of the sporozoite?*

In the mosquito midgut we only observed a small proportion of deformed sporozoites on d12-14. Egress from oocysts does not seem to be impaired since sporozoite are able to efficiently colonize salivary glands. We have now done more infections and also included quantification of the sporozoite shape in the hemolymph on day 16; which are now included in Figure 1G; for direct comparison see graph here:



In the hemolymph almost 80% of sporozoites appear normally shaped while at the same time point in salivary glands only 27% normally shaped sporozoites could be observed. We can't know if those hemolymph sporozoites that are misshaped have been staying in the hemolymph for prolonged periods of time and therefore deform or whether they were deformed before and cannot get into the salivary gland due to deformation.

Minor comments

1. *May be the nomenclature of Pf concavin (Fig. 2) can be changed as it is misleading to say Pf concavin since the protein of Pf concavin is studied in chimera of Plasmodium berghei expressing rather than the gene deletion in P.falciparum.*

Thanks, this was indeed potentially confusing. We have now changed the nomenclature. It now reads concavin(-)$^{\text{Pbconcavin-gfp}}$ instead of Pb complementation and concavin(-)$^{\text{Pf3D7concavin-gfp}}$ instead of Pf complementation. (see lines 212-213)

2. *Can author please mark the apical end of the parasite?*
We now added asterisks to mark the front of sporozoites and ookinetes where applicable.

Overall a very nice work and really enjoyed reviewing it.
Thanks for the generous comment.

Reviewer #1 (Significance (Required)):

The work is more focussed on the gene involved in the maintenance of cell shape malaria parasite but may have interest to other cell biologist as well. The various way and assay the authors have used can be useful for community studying cell invasion.

We thank the reviewer for the constructive and encouraging comments

My expertise is on the cell biology of malaria transmission stages.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Summary: In this study, the authors characterize the function and localization of a Plasmodium sporozoite related protein which they name concavin due to its impact on the characteristic sporozoite shape. In summary, the protein was identified by proteomics analysis of secretion (in combination with prev. studies) and P. berghei concavin knockouts (concavin(-)) were generated to study function in multiple Pb life stages in vitro and in vivo. Fluorescent-tagged Plasmodium lines were generated to localize concavin (in comparison to PHIL1, a subpellicular network related protein; concavin-GFP and PHIL1-GFP)) and assess subcellular mobility (e.g. FRAP). The study is well executed, controlled and written and the findings suggest that concavin is a mobile protein that plays an important role in mammalian infection by maintaining the sporozoites' shape when it enters the narrow salivary glands and proboscis in mosquitoes and during migration through the skin en route to the liver.

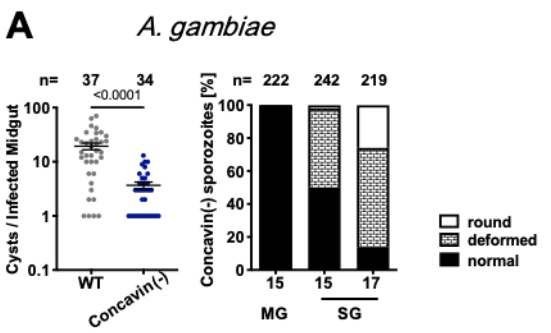
Major comments

The authors provide a comprehensive and detailed study which is well structured. However, they do not consider the already existing literature of this Plasmodial protein and its effect on mosquito-stage P. berghei parasites. Previously, this protein was studied by Ukegbu et. al 2021 Frontiers in Cellular and Infection Microbiology, in which the authors performed a screen and knockout studies of PBANKA_1422900 in P. berghei, which they call PIMMS22 (also the name in PlasmoDB). Ukegbu and colleagues investigated transmission to the mosquito and showed a modest defect in oocyst production, even when mutant ookinetes were injected into the midgut epithelium, demonstrating some role in ookinete-to-oocyst development at the basal lamina (rather than midgut traversal). Ukegbu et al performed localization studies including in sporozoites but did not report a defect in sporozoite shape. However bite back experiments failed to cause patent infection in mice, demonstrating PIMMS22 is also required to infect the mammalian host. The current manuscript by Kehrer et al nicely adds information by providing further evidence of why mammalian infection is blocked - a subset of KO sporozoites have abnormal shape that precludes their normal movement in the mosquito, including through the proboscis, and in the mammalian skin.

The authors should integrate the earlier study into the manuscript including:

- *correcting their statement of identifying a new protein*
- *mention their differing phenotypes regarding oocysts and list any experimental parameters that can be identified to differ between studies that could account for the oocyst differences*
- *consider the gene nomenclature, perhaps harmonising the name concavin with PIMMS22.*

Response: Thanks for pointing to the PIMMS study and saving us some more embarrassment. We only noticed the overlap ourselves after submission of our manuscript and now included the paper in introduction and discussion. After discussions among the authors and upon advice of reviewer 3 we decided to keep the name concavin, since it reflects the phenotype while PIMMS22 essentially refers to a number from a large screen. Importantly, the study by Ukegbu et al used *A. coluzzi* mosquitoes and obtained a more prominent phenotype in oocyst development and complete transmission block in contrast to infections of *A. stephensi* mosquitoes.
We have now added additional data on ookinete conversion and motility (see Figure 1 and also response to Reviewer 3) and confirm the data by Ukegbu et al. who also did not see any significant difference to wild type at these stages.
We also performed a single infection of *A. gambiae* mosquitoes with our *concavin(-)* parasite line. This also confirmed the more drastic effect in mosquito infectivity compared to WT than for *A. stephensi* infections. In addition, the few sporozoites, we found in salivary glands, showed deformations similar to sporozoites developed in *A. stephensi*. This is now included as Figure S4. Albeit this being just a single experiment, considering the match to the prior data of Ukegbu et al., we think it valuable to reporting it as such. Shown here for convenience:



With regard to the concavin(-) phenotype, it is notable that only a subset of sporozoites have the shape defect, which changes over time and parasite location. The reasons for this should be further explained (e.g. could it be heterogeneity of population, sporozoite age, location, maturity etc?).

The data seems most consistent with an effect of sporozoite maturation in the salivary gland as the percentage of deformed parasites and their degree of deformation increases with residency in salivary glands (Figure 1G). But clearly the sporozoite population is somewhat heterogenous with e.g. lengths and curvatures of individual parasites distributed around a mean (see e.g. Muthinja et al., Adv Healthcare Mat 2017). We have now added this to the results and reads "The apparent asynchrony of deformation hints towards an effect of sporozoite maturation on concavin function that affects individual sporozoites to different degrees." (see lines 181-183) and also added to the discussion: "We only saw a portion of *concavin(-)* sporozoites disintegrating, which might be due to the limited observation time and volume during *in vivo* imaging or reflect a heterologous population of sporozoites, some of which are not impacted as strongly by the lack of concavin. Indeed, sporozoites exhibit a distribution of shapes with curvatures ranging from 0.13 to 0.35 mm^{-1} (REF: Muthinja Adv Healthcare Mat 2017), which might indicate different strengths in shape maintenance of the individual parasites and hence a different impact of the gene deletion." (lines 417-419).

In addition, I have a few suggestions which would improve the study.

1. *The authors claim in line 395 and throughout the manuscript that concavin(-) sporozoites reveal for the first time a loss of cellular integrity as phenotypic consequence. However, this is only the case for 20% of concavin(-) sporozoites in salivary glands on day 17, for 70% on day 28 (Figure 1D) and for 30-40% of concavin (-) sporozoites in the skin (line 306). It would be important to discuss why this occurs only in some and not in all concavin(-) sporozoites which were able to enter narrow spaces (SG and skin)?*

We have indeed only observed sporozoites during a certain time interval and we cannot exclude a higher percentage of parasites disintegrating when observed longer. We included more discussion on possible reasons in the discussion, see answer to previous comment and lines 414-419.

2. *Somewhat related to comment 1, the authors performed extensive experiments to describe the phenotype of concavin(-) parasites and demonstrate the physiological effect. However, how concavin acts is still unclear.*

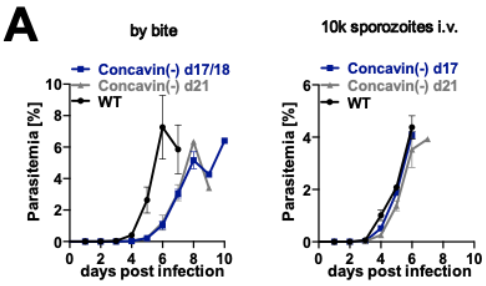
Yes, we totally agree. To get a better idea on the initial function of concavin we are planning pulldown or proximity labelling experiments as a follow up study. We hope the reviewer agrees that it will take at least another three years to get new insight and hence cannot be part of this first study, which describes for the first time a parasite disintegration phenotype during migration in the skin.

3. *P. berghei is a powerful model and some analysis of the orthologous gene in human infecting Plasmodium species would further strengthen the significance of this study in relation to malaria being a disease of humans.*

Of course, *P. berghei* is 'just' a model, but we did do complementation of the knockout with the *P. falciparum* orthologue resulting in a complete rescue of the deformation effect. This to us shows that *P. falciparum* concavin has a very similar function. We therefore do not see the need of repeating infections/ experiments in *P. falciparum* parasites, which is much harder to do than in *P. berghei*. Please note that we and others have followed this path since several years. Key examples are the swaps of P. falciparum TRAP and CSP into *P. berghei*, which yielded parasites with wild type infectivity.

4. *Concavin is essential for efficient transmission of mosquitoes, but anywhere else? The authors demonstrated a delayed prepatency period for concavin(-) sporozoites via bites of mosquitoes, demonstrating a strong defect somewhere between injection of sporozoites and the blood stage. Is it all at the mosquito and skin? To validate their hypothesis that this delay is specifically due to inefficient ejection and migration of sporozoites through the skin, the authors should perform an experiment with dissected sporozoites and inject them intravenously. This would uncouple mosquito feeding to the remaining steps (ie answer whether there is also a liver stage phenotype?).*

The fact that deformed cells are able to invade liver cells in vitro and that the parasites develop normally compared to wild type hints towards a more prominent phenotype at an earlier stage. This of course does not preclude that concaving(-) sporozoites are hampered in getting from the blood to the liver. We have now also injected a total of 10k sporozoites intra venously, and strikingly did not observe any difference between wild type and 2 independent injections of 4 mice with *concavin(-)* parasites. The data below is now integrated in Figure 6A-B as shown here for convenience:



B

	Route of infection	Infected mice	Prepatency (dpi)	Parasitemia day6
WT	by bite	3/3	3	7,3
Concavin(-) d17/18	by bite	5/6	4,2	1,04
Concavin(-) d21	by bite	3/6	4,3	1,18
WT	i.v.	4/4	3	4,37
Concavin(-) d17	i.v.	4/4	3	4.07
Concavin(-) d21	i.v.	4/4	3	3.5

Minor comments

Thanks for these comments, we have now included additional datasets and added more detailed information into the respective figure legends.

5. Significance Section. It would be beneficial for the reader to add a line or two discussing the significance of the finding in regards to application or translation.

We added a sentence to both the abstract and significant section which read "Interfering with cell shape maintenance pathways might hence provide a new strategy to prevent a malaria infection. " (abstract) and "Our work therefore opens up new avenues for intervention by targeting cell shape maintenance." significance. (line 48)

6. Introduction. The authors mention IMC-proteins (line 85), secretion/pore forming secretion proteins (line 90 and 98). It would be beneficial to define those (e.g. in brackets).
Done, now on lines 93 and 116

7. Introduction. The authors mention the protein LIMP (line 103). An explanation of this abbreviation would add more clarity.

LIMP is named after the limping phenotype of the sporozoite when the protein is tagged with GFP, we clarify this now on line 121.

8. Figures. As outlined below in more detail, for some figures and graphs the number of independent repeats per experiment is not clearly described in figure legends. The author should include more detailed information about the number of independent experiments and/or samples used to demonstrate reproducibility of results.

Totally agree, and now done for all.

a. Figure 1B. 32 individual Oocysts counted. Were additional independent

transmission experiments performed and if so how many? Number of repeats has to be mentioned and n=3 is standard.

Yes, now updated in what is now Figure 1E to total of 109 oocysts are counted from 4 independent mosquito infections.

b. Figure 1C; Sporozoite motility assay. No information about number of repeats

this is only an example image series (now Figure 1F) to highlight sporozoite classification used to quantify shape. At least 3 repeat infections were done as reported now in Figure 1 G and H

c. Figure 1E. Number (n) of mosquitoes is not reported.

For each experiment at least 5 infected mosquitos were dissected and midguts or salivary glands were combined into an Eppendorf tube from which small proportion of the released parasites were used for quantifications. now also mentioned in legend to what is now Figure 1G.

d. Figure 1F. How many independent experiments per group? Why are the round sporozoites not included in the graph and at which timepoint was it measured?

also 3, now stated in what is now Figure 1H. Round ones were not tracked because they did not move, this is now stated in the text; line 191 "…and completely round sporozoites did not move at all"
Motility assays are usually done between d17-25 post mosquito infection.

e. Figure 3. Number of independent repeats for each experiment of this figure needs to be stated

3 independent experiments for A and B, now stated in figure legend, while C includes 2 independent experiments D-F are single experiments.

f. Figure 4. Independent repeats need to be reported

In total we reconstructed 6 parasites from two different preps. This is mentioned in the methods section (EM section) and now also in the figure legend

g. Figure 6D. Independent experiments need to be reported

now included in the figure legends, 4 repeats.

9. *Figure 4A. The figure includes two scans with different 2D dimensions for wildtype sporozoites (lengthwise vs 'cut through') but only one image/dimension for the mutant sporozoites. The authors might explain why or include a scan of a different sporozoite orientation to prove the round shape.*

Longitudinal sections are kind of hard to obtain from a round object ;-). Hence, we decided to include the 3D reconstruction of the mutant in Figure 4C. Furthermore,

there are additional images of cells that are only partly rounded in Figure S11, some of which show partly longitudinal sections.

10. Figure 5F. Form the figure it is not exactly clear which parasites are normal and deformed. The authors should indicate deformation with arrows, for example.

All images but not the sporozoite on the top right represent a selection of deformed sporozoites. We noted this now in the figure legend and also added arrowheads.

11. Figure 6C. There is no figure legend for the graph in the middle with describes cells [%] on the y-axis. It is assumed, it shows the percentage of deformed and normal SPZ of intracellular concavin(-) sporozoites, however, this is not 100% clear. The authors should make this clearer.

Thanks for spotting this, we have now added a more detailed description into the figure legend, which reads "Small graph shows the percentage of deformed and normally shaped intracellular *concavin(-)* sporozoites."

12. *Figure 7A and B. The authors should add statistical analysis here as in other figures*.

P-values are now added.

13. *Figure S1. Expression level of concavin in Ookinetes suggest potential importance in that life stage? It would be interesting to discuss this, particular in context with existing literature, see comment below.*

As mentioned above, we have now added a more detailed analyses of the ookinete stage into Figure 1. In accordance to the work on PIMMS22 we did not see a significant difference to wt. Ookinetes developed normally and did not show any deformations up to 30h post infection. See also response to reviewer 3.

14. *Figure S3. I believe the meaning of concavin(-) NS abbrev. is not explained at any part of the manuscript. Please declare.*

NS stands for negative selected, ie resistance marker recycled parasites. It is described in the Methods section, yet to have this clearer we have now added an additional sentence in the results as well as the figure legend.

15. *Figure S8 legend. Scale bar declaration is incomplete. Please correct or delete as it is part of figure itself already.*

Corrected.

Reviewer #2 (Significance (Required)):

A well executed and communicated study that will be of interest to malaria researchers and likely also other cell biologists. The authors should discuss their results in context with the existing literature and carefully navigate any differences to earlier work. Overall, the findings of this study add very nicely to existing literature on

the function of this protein and have impact on further studies of the motility and cell shape of Plasmodium sporozoites in different migratory processes.

Thanks for the generous and encouraging comments

My expertise is in malaria genetics and cell biology including transmission and liver stages.

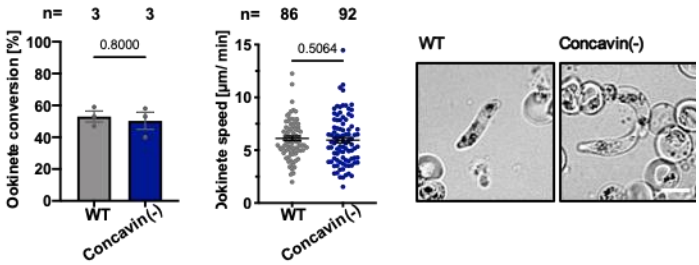
Reviewer #3 (Evidence, reproducibility and clarity (Required)):

1. Evidence, reproducibility and clarity
Summary:
This work provides a forensic characterisation of the role of a palmitoylated protein of unknown function, essential for maintaining the svelte cell shape in malaria sporozoites. Initially using a classic experimental genetic approach the authors investigate its role and localisation using knockout and tagging, revealing it to localise to the sporozoite periphery, between plasmamembrane and inner membrane complex. They also reveal that gene knockout results in a morphological degeneration phenotype in salivary gland sporozoites. The work is set apart by the exceptional analysis of this phenotype in both in vitro, ex vivo and in vivo experiments to determine where they are able to meticulously demonstrate the link between structural integrity, motility, and key steps in the transmission process. The work has a clear narrative and applies an array of cutting-edge imaging approaches in a challenging mosquito/parasite system. Conclusions are well supported and data and statistical analysis are robust throughout.
I just have a couple of major points which can be addressed with changes to text, as well as a number of minor points for the authors to consider.

Major comments:

Ookinete phenotype? Whilst concavelin localises to both ookinete and sporozoite peripheries, and hints of a pre-oocyst phenotype there is almost no mention of ookinetes in stark contrast to the forensic examination in sporozoites. Could the authors comment on whether any deformation was seen in ookinetes and if so whether the deformation changed over time within cultures? Were any experiments carried out on ookinete motility for example? New experiments would not be necessary but some expanded discussion in this area is important.

Response: We have now performed a more detailed analysis of ookinetes in new experiments, also in light of the paper by Ukegbu et. We did not observe a significant difference in ookinete conversion as well as motility compared to wt ookinetes. Furthermore, we observed normally shaped ookinetes even up to 30h post incubation. Results (see below) are now included into Figure 1.



Previous gene description - A paper earlier this year has been published describing a phenotype for this gene Identification of Three Novel Plasmodium Factors Involved in Ookinete to Oocyst Developmental Transition (nih.gov) . Notably there is almost no overlap in the analysis other than defining the gene as important for transmission. Indeed the work focuses predominantly on the preoocyst phenotype so is extremely complementary. The gene has a screening name of PIMMS22, but I don't think precludes the use of a new name now its functional role has been delineated. Consider mentioning the paper briefly in the intro and incorporate fully into the discussion. If no data is available to address the ookinete questions above, then referencing here may be useful for that too.

We unfortunately missed that at the time of writing (paper was largely written in 2020) but we naturally included it now in the manuscript. Our ookinete data as described above recapitulates to the previously published results as did an infection of Anopheles gambiae mosquitoes (new Figure S4).

Minor comments:

Introduction - excellent background to sporozoite biology, but add a few sentences to note that key structures and processes are conserved across other malaria zooites.

We added two mentions about other extracellular zoites into the introduction (lines 86 and line 95)

Line 109- 114 The background here is a little confusing as it starts by highlighting importance of identifying surface proteins and the fact concavin came out of a screen for secreted proteins. It ends up clearly not being surface or secreted protein, so I would simplify this section just highlighting that it came out of a sporozoite proteomics screen and perhaps other reasons it looked interesting.

We deleted the section of how we found the protein, indeed kind of irrelevant how we got to it. We will report the proteomics study in the future.

Line 115 - clarify the delineation of previous and current work. Perhaps something "Here we..."

Did as suggested.

131 "expressed highest" doesn't sound quite right, should be changed to something like "to expressed at highest abundance" or "expression peaks in..."

Fig S2 legend states palmitoylation site is C terminus, but alignment suggests N. Also L136 says N, and L376 says C. Should they all be N?

Thanks for pointing this out. It should of course be called N- terminus. It is now corrected in the text of Figure S2.

Figure 1E seems redundant and displays same data as D (which I prefer). Figure 1F shows data for deformed but not round sporozoites. Do they exhibit any forward motility? Do round sporozoites display any processing on the spot, or spinning (perhaps a video if so?)? This would indicate they remain alive but uninfectious purely down to inability to move forward.

We have now removed Figure 1E (also to make the ookinete data fit into Figure 1). Round cells are indeed unable to move. Therefore, we have not included them into the figure. We have now included an additional sentence into the text.

Fig 2c add a note on the inset salivary gland images in the legend (not mentioned). Also add scale bar to the insets. There is signal in both (and stronger signal in the Pb comp?) but its not completely obvious what the message is without any mention in legend. Insets should be bigger too (tighter crop?).

The idea behind the images is to show that SG are indeed colonized in the KO and is now mentioned in figure legend. Scale bar is added.

L269 - Clarify this sentence. Could you also not confidently see deformed sporozoites or just rounded? Were there 10-20% unclassifiable and therefore possible deformed?

Correct, we added “deformed or rounded” to the sentence, especially minor deformation cannot be seen; now line 347

Morphology of nucleus - Could the authors state whether the deformation of the nucleus is altered (from it notably thin morphology seen in sporozoites) and whether it is associated with deformation and break points? In other stages constriction of nucleus is important for navigating small spaces. Perhaps some discussion of this work is relevent https://doi.org/10.15252/embr.201948896

This is a great comment and we indeed just wrote a grant where part of the goal is to do correlative light and electron microscopy to elucidate what happens to breaking sporozoites. Within this we will also look at the cellular organelles including mitochondrion and nucleus. Nuclei don’t look all alike in sporozoites if judging from Hoechst signals. With the current dataset we don’t feel confident to make much of a statement.

L375 and following. I am not sure I follow the logic of it being more likely to be linked to PM? Whilst we know many IMC proteins are anchored to the subpellicular network, and thus immobile, would one associated with IMC by palmitoylation be expected to be?

We interpret the super-resolution microscopy images such that concavin is closer to CSP than to Phil1, hence we assume (but of course don’t know) that it is more likely

to be found either on the inner side of the PM or the IMC but facing the PM rather than the SPM. We added a reference to the results from figure 3C right to the start of the discussion and also referred to other figures in this first paragraph for consistence and easier cross reading.

Importantly, the palmitoylation mutant experiment demonstrates that it is not required for the peripheral localisation. This should be discussed, and strongly suggests that it binds to an IMC or PM protein. As an aside note, identification of complexes would be a really exciting next step in future work, and pulldowns might be best carried out using in vitro cultured ookinetes or tachyzoites?

We totally agree and have this planned as the next logic steps. Will be tough to do IPs from sporozoites, but we will try, also will try BioID of concavin. We strengthened the discussion of how concavin could work and that palmitoylation does not confer localization just by itself (lines 436-450).

Was an attempt made to tag at N terminus? Although it would have disrupted the Palmitoylation site, it may have been interesting to compare the N-terminal vs C terminal location with STED, to test the IMC spanning hypothesis.

Great idea. We will include this in a follow up study.

L400 and following. I am also strongly reminded of the degenerative ookinete phenotype observed in PDEd KO P. berghei. These parasites also undergo a progressive change from slender ookinetes to deformed and then round. There are some interesting parallels in the IMC disruption, and impeded motility (with intact motor) with rounded forms processing on the spot https://doi.org/10.1371/journal.ppat.1000599 Could be worth discussion.

Thanks, this is indeed a great paper that fits perfectly and we added a few lines to the discussion, lines 463-476, which read "In ookinetes deletion of the phosphodiesterase PDEδ, which breaks down cyclic nucleotides such as cGMP, revealed a remarkably similar phenotype to deletion of concavin in sporozoites. The *pdeδ(-)* parasites formed normally shaped ookinetes, which then progressively rounded up, where not capable of migrating across the midgut epithelium and largely failed to establish a mosquito infection (Moon et al., 2009). Single section transmission electron microscopy showed the partial absence of the IMC. How either PDEd or concavin contribute…".

L423, again unless the authors explain the link with secretion, it may be clearer to leave out the statement regarding its identification.

We deleted the statement

Reviewer #3 (Significance (Required)):

2. Significance

The work presents an important conceptual advance in both the key role of a malaria

protein of previously unknown function as well as a deeper understanding of the relationship between cell shape, motility and interactions with distinct host environments. Apicomplexan parasites are highly polarised and their morphology is underpinned by unique structures including the inner membrane complex and an associated actomyosin motor. Whilst several protein families are known to contribute to these structures, concavin represents a new class conserved in all apicomplexans. Its loss seems to be particularly disruptive in sporozoites, and the detailed analysis demonstrates both that this structural integrity is vital for supporting cell migration - and critically this is most important at the key boundaries of salivary gland secretion and skin migration of sporozoites.

The combination of technical approaches taken to examine the phenotype in sporozoites, is also exemplary and will be of significant interest to those working on difficult to image vector-borne pathogens.

The work will be of great interest to those studying host pathogen interactions, cellular motility systems as well as those working on invasive stages of malaria parasites.

A previous knockout of this gene has been published but did not identify the key morphological sporozoite phenotypes nor the specific barriers to transmission encountered. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8005625/
As such I do not feel this has any negative impact on significance of this particular work.

We also thank reviewer 3 for the generous and encouraging comments

My field of expertise is in molecular parasitology, particularly in experimental genetics of malaria parasites, as well as motility and invasion of malaria parasites.

1st Editorial Decision 9th Feb 2022

Dear Prof. Frischknecht,

Thank you for the transfer of your revised manuscript to our editorial offices. I have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees fully support publication of your study in EMBO reports.

Before we can proceed with formal acceptance, the manuscript needs formatting according to our journal style. Please carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your final revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

For more details, please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation

Please consult our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

3) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

4) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also for potential EV figures and all those in the final Appendix). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please also note our reference format:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) For microscopic images, please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

11) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement'.

12) Please remove the significance statement from the manuscript text or fuse this with the abstract.

13) Please provide the abstract written in present tense and with not more than 175 words.

14) Please remove the highlights section from the manuscript text. These can be used for the bullet points (see below).

15) Please include the funding information into the acknowledgements. Please enter all the funding information also into our submission system and make sure this is complete and similar to the one mentioned in the manuscript text file.

16) Please name the movie files 'Movie EVx' and change the callouts accordingly. Moreover, we need a legend for each movie file. Please provide this as a text file and ZIP it together with the movie file, and upload these as one folder. Finally, please remove the mention of the supplementary movies from the manuscript file.

17) Please include Supplementary Table 1 into the Appendix (naming this 'Appendix Table S1'). Finally, please change the callouts and remove the mention of the supplementary tables from the manuscript file.

18) One Excel table with 'raw data' has been uploaded, which seems to be source data. We would need these separated and named after the figure or panel these data are shown (e.g. Source data Figure 1). Or, these tables need to be included into the Appendix file, named 'Appendix Table Sx', provided with a legend and called out in the text.

19) Please fix the typos indicated by referee #1 and carefully proofread the final manuscript text.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- three to four short bullet points highlighting the key findings of your study.
- a schematic summary figure (synopsis image) in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels that can be used as a visual synopsis on our website.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

Clarity / figures: All previous comments about the integrity of the figures with regards to statistical analyses, reports of number of experiments and technical repeats were addressed adequately by the authors.

Remarks to the Author:
The authors have made all the relevant and most important changes and have dealt adequately with the concerns raised about discussing their findings in context with the existing literature. Gratifyingly, they added a mosquito infection experiment (S4) and could demonstrate similar results to Ukegbu et al.'s study. The authors also included the suggested experiment (6A/B) to inject sporozoites intravenously which confirmed concavin's role during migration in the skin as previously suggested by the authors. They also discussed appropriately why only a portion of concavin(-) sporozoites may have disintegrated which will be of benefit for the reader.

There are some spelling errors:
line 46: strictures, line 128: strictures, line 447: strictures, line 1165: double period . .

Referee #2:

The authors have done a great job of fully addressing all of my previous comments raised. There is also now significant new experimental data which clarifies some additional points, and further enhances the work.

It is an excellent piece of work, and recommend publication.

Referee #3:

The authors have addressed most of my comments and I have no further question.

Medizinische Fakultät Heidelberg | Im Neuenheimer Feld 344 | 69120 Heidelberg

To:
Dr Achim Breiling
Editor
EMBO reports

Dear Achim,

Thanks for overseeing the review of our manuscript and for accepting to publish it in EMBO reports. We have now (hopefully) completely adapted it to EMBO reports style and provide the files including a final "clean" manuscript file with bullet points, 2 sentence summery and abstract of less than 175 words as well as the 7 figures as tiff, 3 EV figures, 5 EV movies and an appendix with the rest of the supplementary information as well as source files for the figures where they apply. Also we include an author checklist.
I would like to submit a suggestion for a cover, as we have many artistically appealing images from our parasites but would like to wait until hearing back from you about our second manuscript on myosin function in Plasmodium as we would choose a different image if the two papers could be published back-to-back.

Please don't hesitate to contact us, should we have missed something.

Sincerely, Yours

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1st Revision - Editorial Decision 8th Mar 2022

Dear Prof. Frischknecht,

Thank you for the submission of your revised manuscript to our editorial offices. Before we can proceed with formal acceptance, I have these editorial requests I also ask you to address in a final revised manuscript:

- Please remove the bullet points and the summary blurb from the main manuscript text. I have saved this separately and will forward it to our publisher.

- Please make sure that the figure panels are called out in a sequential manner (alphabetical, as they show up in the figure). Presently, Fig 2E is called out after 1F. In case, please change the order of panels in the figure.

- Please provide separate (and sequential) callouts for all the figure panels, including EV and Appendix figures. Presently, there are no separate callouts for the EV figures and most Appendix figures.

- It seems that author Sebastian Weber is missing from the author contributions. Please check.

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main and EV figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant.

- Please add scale bars of similar style and thickness to the microscopic images (also in the Appendix), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently, some panels do not have scale bars (e.g. 3D/E, S4E or S9) or the size is not defined (e.g. S6).

- In the Appendix file, please move the two panels of Figure 2 on one page with the legend on the bottom. It is fine to have one figure per page in the Appendix. Please then update the TOC (mentioning just Appendix Fig. S2 for page 3).

- In Appendix Fig. S5C the first two gel pictures ('Transfection' and 'Limited dilution') look identical. Please check and/or explain.

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and add a statement declaring your competing interests. Please name that section 'Disclosure and Competing Interests Statement' and add it after the acknowledgements section.

- Please add a 'Data Availability' section (DAS) to the manuscript, also if no large datasets have been submitted to a public database. Please state there 'No large primary datasets have been generated and deposited'. Please place this after the Methods section, before the acknowledgements.

- On page 9 a 'Supplementary Data Table S2' is mentioned. What does this refer to? Please check.

- Please format the references according to our journal style (we need et al. for publications with more than 10 authors). See also:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Best,

Achim Breiling
Senior Editor
EMBO Reports

The authors have addressed all minor editorial requests.

Prof. Friedrich Frischknecht
Heidelberg University
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Germany

Dear Prof. Frischknecht,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Yours sincerely,

Achim Breiling
Editor
EMBO Reports

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Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

EMBO Press Author Checklist

Corresponding Author Name: Friedrich Frischknecht	
Journal Submitted to: Embo Reports	
Manuscript Number: EMBOR-2022-54719V2	

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Reporting Checklist for Life Science Articles (updated January 2022)
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures
1. Data
The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Appendix Table S1

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods

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Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Materials and Methods

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgements

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Experiments independently done by different students
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	All data is included in the manuscript
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

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Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

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The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
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Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	