

PfPPM2 signalling regulates asexual division and sexual conversion of human malaria parasite *Plasmodium falciparum*

Corresponding Author: Dr Pushkar Sharma

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)

The study by Rawat et al have an interesting study showing the critical role of Protein Phosphatase PfPPM2 in both asexual division and sexual conversion in the malaria parasite *Plasmodium falciparum*. Through phosphoproteomics done on PPM2 depleted parasites, they have shown that the loss of PfPPM2 affects the phosphorylation of histone H3 and its variant H3.3, as well as the translation initiation factor eIF2 α . This regulation is essential for maintaining proper chromatin structure and protein synthesis, which are critical for cell division. The major finding of the study is that they have elaborated the role of PfPPM2 in regulating the sexual commitment of the asexual blood stage parasites. PfPPM2 specifically dephosphorylates Heterochromatin Protein 1 (HP1) at S33, which prevents its interaction with H3K9me3, leading to the derepression of the transcription factor AP2-G, which is essential for sexual differentiation.

Overall the noteworthy study especially the correlation of PPM2 with HP1 which is the strongest part of the data. Though the authors mention about asexual division but this part can be improved if they want to add asexual division in the title. Some high- resolution images about the defect in the asexual division like with tubulin and centrin could be provided to get clear picture on that aspect of biology. It is also important to get few more details about the correlation with AP2G as this part is not well demonstrated in the study. If the authors have the PPM2tag parasites then pulldown data may be added to strengthen their paper but if it required too much time then can be avoided.

General comments

1. for the general reader, more details would be good around PPM2 regarding the fact that it is a chimeric protein phosphatase containing two PP2C domains with homology to PTC2 and PTC3 of *S. pombe*.
2. The authors present data on sexual conversion but have they analysed the sex ratio of male vs female in these knockdown parasite. Is there any idea which type of gametocytes are increased?
3. Although the study mentions the role of HP1 in var gene regulation, it does not delve deeply into how PfPPM2 affects the expression of these genes, which are critical for immune evasion. Some discussion on it.
4. There is defect in merozoite number but is anything known about the parasite invasion as they found defects in IMC. Some comments on it

5. While the study identifies PfPPM2 as a key regulator, it does not identify the kinase responsible for phosphorylating HP1 at S33. Any thoughts on it. Also if the PPM2 is regulator of cell division then how does it work with PP1 which is key regulator of cell division. Some discussion on it.

Comments on Figures

Fig legend 1C – says the right panel has been done by flow cytometry but the left by counting Giemsa stained smears.

Looking at the figure, it seems that the left graph is for 3D7 and the right graph is for NF54. Why were the 2 quantified differently? The figure legend should explain that the graphs are for the 2 different strains.

Fig legend 1E – says “and is localized in close to nucleus and/or in close proximity (DAPI)”. Remove the first “close to”. Also, how big are the scale bars in these images? In the centrin images at the bottom, the letter “l” in DAPI is green where the rest of the word is blue. Change it to blue.

The scale bars are difficult to see in the top of panel 1F. Also, the tubulin staining in the control panel doesn't look very spindle-like. Do they have a better or higher resolution image?

Fig 2A – explain in the legend what the different colours mean for the proteins highlighted in the graph – in main text

described as 3 major families

For 3C, do the authors have any images that would support the graph? Same for 4B

Fig 5B, provide scale bar.

Fig 5C – it looks as though the size of the scale bars is written above the scale bars but it is very difficult to see. Remove the writing from the image panels and give the size in the figure legend instead. Also, a statistical comparison could be given between HP1 and S33D for completeness as HP1 is the control

Fig 5D – statistical comparison could also be given between HP1 and S33D

Fig 5F is not mentioned in the text

Fig 6A – scale bars are very difficult to see and no size is given in the legend. The data in this figure isn't really surprising and doesn't really add much to the story. If S33 has been mutated to alanine, it's no surprise there's no pS33 staining. This could be added to supplementary. Equally, if the key thing here is that S33A mutants have more gametocytes, this message should be clearer in the text rather than the pS33 stain (lines 300-303).

Some comments on the text

Line 32. A more up to date reference is needed here other than Cowman et al. (e.g. Guttery et al, 2022).

Line 38 – the statement “the function of most protein phosphatases remains unknown.” Is not strictly true. Guttery et al. 2014 provided evidence of the key functions for many of the *P. berghei* phosphatases and Morano et al. 2023 showed that the pseudophosphatase PPP8 is required for the maintenance of *P. falciparum* basal complex integrity. Please correct this.

Line 61 – Please correct (Prommana et al) as no date is given.

Line 87 and Figure 1F – what do the authors mean by “diffused tubulin staining”? Please clarify this. To my mind, diffused means that tubulin shows staining throughout the parasite, but the figure shows only one tubulin in the + panel of Figure 1F, whereas the graph shows an increase when GlcN is added. This data would be better represented as the number of parasites positive for tubulin staining.

Line 143 – the implication that PfPPM2 may negatively regulate PK4 activity is highly speculative. Unless hyperphosphorylation of PK4 can be shown in PfPPM2 depleted cells, I would advise removing this statement.

It is good that the authors have confirmed previous studies showing a decrease in gametocytogenesis in absence of PPM2. Have the authors considered also seeing whether the female:male gametocyte ratio also changes to 1:1, as seen in *P. berghei*?

Please check the labelling for genes in the Supplementary RNA-Seq data. Many annotations are wrong. For example, AP2-G (PF3D7_1222600) in the table for NF54 is labelled as “unannotated transcript”.

Line 331 – Please alter the statement “HP1 at S33 is regulated by PfPPM2”. The authors have only shown indirect evidence of this.

Methods – For qRT-PCR, the authors state that 18S rRNA was used as the HKG, but the supplemental methods state Actin and 18S rRNA was used. Please be consistent. If only 18 rRNA was used, I strongly suggest using an additional HKG as this is common practice.

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

Review of Rawat, et al. NCOMMS-24-59283-T

The manuscript by Rawat and colleagues evaluates the role of PfPPM2 signaling during the blood stages, asexual and sexual, of *Plasmodium falciparum*. The study combined genetics, phosphoproteomics, and transcriptomics to demonstrate the multitude of potential functions for PfPPM2. The findings regarding the relationship between PPM2 and HP1 hypophosphorylation are the strongest and most specific finding. The authors provide a series of convincing experiments to demonstrate the importance of this relationship.

Major

1. Figure 1: The description of the localization of PfPPM2 as “present in punctate structures near the parasite nucleus during schizogony, particularly in mid to late schizonts, with some presence in the cytoplasm” is unclear. The immunofluorescence shown in Fig. 1D for schizonts, shows bright punctate staining that is primarily cytoplasmic with fewer, smaller foci of staining that overlap the nuclei. Cross-sectional imaging would help to improve these localization studies. The claim that spindle microtubules are lost in the knockdown is also not well-supported by the images. The +GlcN parasites have multiple nuclei that have divided. Therefore, they must have had spindle microtubules at some point. The associated figure S3D also seems to not match with the phenotype. The staining in S3D shows diffuse localization of IMC1g but normal plasma membrane localization/partitioning with MSP1. The parasites that are formed in the PPM2 knockdown are able to egress and reinvade. This seems like if the IMC were so disrupted that the parasites would not be successful during egress and invasion.

2. The defects during asexual development seem myriad, including fewer merozoites, abnormal nuclear division, abnormal

IMC, decreased protein production, and increased phosphorylation of multiple proteins in multiple processes. It would be helpful for the reader to dissect which of these processes are direct and which are many steps downstream of the PfPPM2 defect. The decreased expression of AP2-G decreases sexual conversion, but it might also increase asexual replication. However, given the widespread defects it is hard to know how this balance plays out.

Minor

1. Line 7 of abstract – “but” should be “by”
2. Line 41, 49, 61, 63, 64 –grammatical errors.
3. Line 64 and Fig 1A are incorrect. Glucosamine does not bind the glmS ribozyme. Glucosamine is converted to glucosamine-6-phosphate, and that is what binds to the ribozyme.
4. Figure 1 seems messy, with differently colored scale bars, some scale bars with lines at their ends, other scale bars without end lines, and font color switches in “DAPI”. This requires some improvement in presentation.
5. Figure 2C has some overlapping text in the AA sequences.
6. Figure 3A has multiple different fonts and some of the AA sequences looks like it is cut off. 3C should also include the raw conversion rates for the gametocyte inductions.
7. Line 242-244 – Why is this a preliminary result? If the result is reproducible, it should not be preliminary. If it is not reproducible, it should be removed from this section. Figure S6A: the top blot needs to be labeled with which antibody is used, presumably anti-HA.

Reviewer #4

(Remarks to the Author)

In this study, Rawat et al. describe the role of PfPPM2 during asexual and sexual development in *P. falciparum*. They further show PfPPM2 removal perturbs the parasite's phosphoproteome leading to increased phosphorylation of several proteins and the authors followed up on Heterochromatin protein 1 (HP1) in detail. They have clearly demonstrated that S33 residue on HP1 is crucial for function and altering to a phosphomutant (A) or phosphomimetic (D) leads to either overcommitment to sexual stages or deleterious effects on asexual growth. Overall, the authors have performed a challenging study and have shed light on the role of phosphorylation on parasite HP1 function. However, to conclude several of their mechanistic hypothesis, additional experiments are required including various experimental controls.

Major concerns:

- Avoid using green and red together in IFA images and in graphs. They should be recolored for color blind friendly visualization.

-Line 70: From the study it appears PPM2 is not essential for completing the asexual life cycle (unless there is some leaky expression from the glmS system) and growth rate is attenuated due to reduced merozoite production. It will be more useful to represent the actual fold change in parasitemia at each cycle and compare to WT instead of normalising WT to 1.0 (Fig 1C). Additionally, rather than performing the time course study and classifying the stages as ring/trophs/schizonts (FigS3A), it would be more useful if the number of DAPI/Hoescht stained nuclei were counted over the time course to determine if the reduced merozoite count is due to fewer number of nuclear divisions or a slower cell cycle.

-Give more details in the methods for how invasion rate was determined in PfPPM2-HA-glmS3D7 (SF3C) since the GlcN schizonts make fewer merozoites.

-Line 79 and Fig 2E The observation is confusing and the relevance is not clear : ‘PPM2 is present near the nucleus with some presence in the cytoplasm’: Near the nucleus would imply localisation in the cytoplasm or another organelle. Higher resolution images (U-ExM)/ super resolution or organellar fraction is required to state if location changes during development.

-Line 86 and Fig1F: IFAs don't have enough resolution to conclude if there is loss of MT spindles. Higher resolution images should be included to justify this statement.

-Line 88 and FigS3D. Presenting diffused staining of IMCg is not sufficient to state formation of IMC is disrupted. Either additional markers of IMC should be presented or TEM should be performed to demonstrate impaired IMC biogenesis.

-Line 134 (and Fig S5C): The increased S59 phosphorylation in eIF2alpha is very dramatic. Was this site discovered in phosphoproteomics analysis when PfPPM2 was depleted? If not, please speculate why.

-Line 139 (and Fig S5D): If protein synthesis is inhibited, the defect on parasite growth should be very pronounced. The quality of blot for the Puromycin incorporation assay is low and the assay should be performed at least two different time points of the parasite life cycle to confirm effect on protein synthesis.

-Line 197 pS-33HP1 (Fig 3B) blot is not convincing enough. If there are additional replicates, please show a blot with clearer pS33 band and include a loading control since HP1 levels are different.

-Line 228: Reword sentence: 'These observations suggested that PfPPM2 may prevent the association of HP1 and H3K9me3 with heterochromatin...' this suggests PPM2 prevent association of H3K9me3 with heterochromatin which is inaccurate since H3K9me3 is a heterochromatin mark.

-Line 228: Since HP1 antibodies are available to the authors (as shown in Fig3B), overexpression of HP1 should be confirmed using antibodies targeting HP1 which would detect both endogenous HP and HP1-GFP tagged protein.

-Fig4A and B nicely demonstrate that HP1 overexpression lines (although western blots should be performed to confirm this) rescue the PPM2 depletion phenotypes. The authors do not discuss why this might be the case. How does increased levels of HP1 alleviate PPM2 defect?

-Line 227: Fig5C does not display Stage IV and V gametocytes. Cells look like Stage III.

-Fig 5E – what stage was experiment performed on? If >50% of the parasites are gametocytes (S33AHP1 line), it is possible HP1 does not bind to H3K9me3 in early stage gametocytes and reduction in S33A-HP1 observed is due to the life cycle stage of the parasites surveyed rather than a property of S33A-HP1. This should be tested by using HP1-GFP line and performing the IP in Stage II/III gametocytes.

- Blots in Fig5E don't look clean enough for accurate proteins quantification (S33D IP and WB , top panel). Moreover, it is important to show input levels of HP1-GFP protein for all lines. This is critical since significant amount of H3K9me3 is pulled down in the NF54 line in the absence HP1-GFP construct (lane1, IP panel).

-Line 261 and FigS8B. While it is clear WT HP1-GFP is highly expressed and stabilised in the presence of Shield-1, it is difficult to conclude there is overexpression of S33A in the presence of Shield. The levels of S33A looks similar +/- Shield.

-Line 321: The statement PPM2 is dispensible for asexual growth and therefore Pb and Pf PPM2 have diverse function is not accurate. Plasmogem analysis (<https://plasmogem.umu.se/pbgem/>) demonstrates PbPPM2 ko parasites grow slower which is comparable to the phenotype observed in this work (Fig1C)

Fig 6. From the FigS8B, it appears the S33A-DD line behaves similarly in the presence or absence of Shield (protein levels and growth rate). Therefore, the rationale of using Shield in the experiments shown in Fig6A is not clear.

Minor comments:

Fig 2C – add error bars to growth curves. Indicate on graph when GlcN was added.

Lines 168-184: Since the authors performed extensive analysis of RNA, proteome and phosphoproteome it would be useful if the authors presented /discussed effects of PPM2 are specifically on the phosphoproteome (without affecting protein levels) and which changes are due to differences in the proteome.

Fig 3E : number of replicates and error bars should be included.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed my comments and provided expansion microscopy images for tubulin and centrin. This is more clearer in the present manuscript. They have scored the tubulin signal in the mutant versus control but it would have been good to have centrin the same in Fig1F to have complete quantification.

It is appreciated that authors have provided additional images at different stages and also performed U-ExM, please could the authors clearly label which stage of schizont development is which in Figure 1E since they mention "different stages of development". Please be clear what timepoint each row is. Please also make this clear in the text and methods.

Overall most comment were addressed and provided valid discussion.

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

The revised manuscript by Rawat addresses some of the concerns from the previous reviews. As noted previously, their findings regarding the relationship between PPM2 and HP1 hypophosphorylation are the strongest and most specific finding. The microscopy data to support their conclusions remains somewhat weaker than the proteomic and more functional studies. This is a minor aspect of the study, so this weakness is less important. The authors continue to provide a series of convincing experiments to demonstrate the importance of the relationship between PPM2 and HP1.

Major

1. Figure 1: The authors should be commended for their use of U-ExM. This is a major improvement from the images in the first submission. While the localization claims are not critical for the study, they could be displayed more clearly in the figure. It looks like the U-ExM images are maximum projections, which really is not the correct method to show localization or, more specifically, to show co-localization. The authors utilized z-sections to obtain the data, but the axial localization differences are lost in maximum projections. In addition, all images should be treated with the same settings – laser power, gain, and post-processing. This allows the reader to know the relative amount of the protein present. For example, the dim staining may just be background given that a negative control (untagged parasite) is not shown for the mouse anti-HA antibody. In 1F, the graph is a nice attempt to provide some quantification of the phenotype. However, the meaning of “parasites with normal tubulin staining” is unclear. In order for this quantification to be meaningful, highly synchronized parasites with the same number of nuclei should be compared. The meaning of the graph is lost somewhat without more precise clarification. Also, are these parasites from the first cycle after knockdown or the second?
2. Sup Fig 2D – The statement that there was no difference in invasion is a little hard to understand. If fewer merozoites are formed then how is the rate of invasion the same for knockdown parasites. The decreased number of merozoites is likely the source of the replication defect shown in fig 1. I guess that the sup fig 2d is the first cycle, and the replication defect is not fully realized until the second cycle. If there were no difference in the number of new rings formed, then there would be no growth defect. This should be clarified. Perhaps the best thing would be to show the relative invasion for the second cycle instead. Lines 65-71 are somewhat confusing as currently written.
3. Sup Fig 3D and 3E. I mentioned this in the previous section. While I appreciate the comments from the authors, it does not make sense that invasion is “normal” when both IMC1g and GAP45 staining are so abnormal. The manuscript that is mentioned in the rebuttal letter about IMC1g shows relatively normal GAP45 staining during segmentation (see Fig 2E in Cepeda Diaz mBio 2024). Again, I think that this is not the major focus of the study. However, the data of abnormal GAP45 and normal invasion are not entirely consistent. I am not sure that this needs to be reconciled, but it is worth a bit more description of this putative inconsistency in the text than is currently present.

Minor

1. Line 38 – as noted by one of the other reviewers, this statement should be modified to reflect the data from the P. berghei study. The loss of PbPPM2 caused a “significantly slow” growth phenotype. The current version suggest that there was no growth defect and should be clarified to aid the reader’s understanding.
2. Line 75 – the term Ultra Expansion Microscopy is incorrect.

Reviewer #4

(Remarks to the Author)

The authors have performed additional experiments and addressed majority of my concerns. However, a few matters require clarification before publication:

- 1.Fig1E: mention which lifecycle stages are examined (hpi?) PfPPM2 has an interesting staining pattern and could be localised at the MTOC in late-stage parasites (bottom 2 rows). The authors should provide ‘zoomed’ images of PPM2 localisation in these parasites. This could explain the fragmented tubulin staining in phosphatase depleted mutants in Fig 1F
- 2.Fig1F: mention how many parasites were counted.
- 3.FigS6A: The additional western blot with HP1 antibody shows a very minor increase HP1 levels in the over expressing line (compare endogenous HP1 level 35kDa to HP1-GFP at 60kDa). The authors should discuss whether such a small increase in HP1 protein levels actually rescues the phenotype or the OE plasmid might change HP1 properties whether it is regulation, stability etc.
- 4.Although the authors claim that taken together their pulldown data in Fig 5E and immunofluorescence in Fig6A give evidence HP1-S33A-GFP is does not bind H3k9me3, this evidence is circumstantial since the pull down is performed in different developmental stages. If it is challenging to perform the pulldown experiments in stage II gametocytes, at least this caveat should be discussed.

Minor comments:

1. Maintain consistency in error bars. Some graphs have bi-directional and others are top (even when showing the same assays e.g Fig1C vs Fig4A
- 2.This could be due pdf conversions, but will be useful to check space between panels in IFA images.
- 3.Correct annotation of gene- either use AP2G or ap2g in Fig6C

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

The re-resubmission has addressed all of my previous concerns, more or less. The authors have done a good job trying to reasonably answer my previous concerns. I may not agree with all of their answers, but these are more stylistic concerns than questions about the validity of the data or the conclusions.

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Point-wise response to reviewer's comments: Rawat et al NCOMMS-24-59283-T

Please find the response to queries raised by the reviewers indicated in **blue** font.

Reviewer #1

"Overall the noteworthy study especially the correlation of PPM2 with HP1 which is the strongest part of the data. Though the authors mention about asexual division but this part can be improved if they want to add asexual division in the title. Some high- resolution images about the defect in the asexual division like with tubulin and centrin could be provided to get clear picture on that aspect of biology. It is also important to get few more details about the correlation with AP2G as this part is not well demonstrated in the study. If the authors have the PPM2tag parasites then pulldown data may be added to strengthen their paper but if it required too much time then can be avoided."

The reviewer has highly appreciated the reported studies and has raised a few queries which we have addressed below. In brief, we report results from Ultra Expansion Microscopy (U-ExM) for centrin and tubulin localization which has strengthened the conclusion related to asexual division. In addition, we have provided new data in response to the queries. Unfortunately, we are unable to perform the pull down at this time but feel that phosphoproteomics data provides key information on PfPPM2 targets.

General comments

"1. for the general reader, more details would be good around PPM2 regarding the fact that it is a chimeric protein phosphatase containing two PP2C domains with homology to PTC2 and PTC3 of S. pombe."

In the initial study (Mamoun et al, 1998), it was mentioned that PP2c/PPM2 may possess two phosphatase domains but subsequent analysis (Guttery et al, 2014; Wilkes & Doerig, 2008) suggested that PPM2 has single phosphatase domain but has insertions in it which makes it larger in size than a conventional phosphatase domain. We have mentioned this in the revision (**page 4, line 52-56**).

"2. The authors present data on sexual conversion but have they analysed the sex ratio of male vs female in these knockdown parasite. Is there any idea which type of gametocytes are increased?"

Since we did not have antibodies against proteins specifically expressed in male and female gametocytes, we could not measure their ratios.

"3. Although the study mentions the role of HP1 in var gene regulation, it does not delve deeply into how PfPPM2 affects the expression of these genes, which are critical for immune evasion. Some discussion on it."

Since the focus of the study was on the role of PPM2 on parasite development and the involvement of HP1 in this process, we did not follow the var gene regulation. However, interestingly, the RNAseq data suggests that the expression of several Var genes is altered upon PfPPM2 depletion, which is possibly due to the deregulation of HP1. We have indicated this in the "Discussion" (**page17, line 366-372**) section and have highlighted these genes in Supplementary Fig. 9. In addition, we have

now assessed the expression of VarB expression, which is regulated by HP1 and found that it was down regulated upon PfPPM2-depletion (**Supplementary Fig. 9C**).

"4. There is defect in merozoite number but is anything known about the parasite invasion as they found defects in IMC. Some comments on it"

We have reported in **Supplementary Fig. 2D** that no significant defects were found in invasion upon PfPPM2 depletion. Despite disruption in IMC formation, the lack of defects in invasion of PfPPM2 were surprising. However, recent studies revealed that IMC1g-depleted parasites also do not show significant changes in egress and reinvasion (Cepeda Diaz et al, 2023; Liu et al, 2024). We have mentioned this in the text (**page 6, line 96-99**). It is possible that (~50%) parasites that had proper IMC invaded RBCs successfully.

"5. While the study identifies PfPPM2 as a key regulator, it does not identify the kinase responsible for phosphorylating HP1 at S33. Any thoughts on it. Also if the PPM2 is regulator of cell division then how does it work with PP1 which is key regulator of cell division. Some discussion on it."

The reviewer is probably referring to PP1 related studies (Paul et al, 2020), which indicate that it regulates both division as well as egress of the parasite. Since its targets-identified by phosphoproteomics- are very different from the ones of PfPPM2, it is very likely these PPs operate via independent mechanisms. At this point, we do not know the identity of the kinase involved in HP1 phosphorylation at S33. One of the possibilities is that one of the kinases involved in cell division may play this role. We have included these points in the **Discussion (page 19, line 402-407)**.

Comments on Figures

"Fig legend 1C – says the right panel has been done by flow cytometry but the left by counting Giemsa stained smears. Looking at the figure, it seems that the left graph is for 3D7 and the right graph is for NF54. Why were the 2 quantified differently? The figure legend should explain that the graphs are for the 2 different strains."

We have assessed parasite by counting Giemsa-stained smears and flow cytometry to validate growth defects by using two independent methods. For 3D7 we have used parasite counting from smears but with NF54 we have done both methods, which revealed similar results but we have reported only flow cytometry. We have modified the figure legend as suggested by the reviewer.

"Fig legend 1E – says "and is localized in close to nucleus and/or in close proximity (DAPI)". Remove the first "close to". Also, how big are the scale bars in these images? In the centrin images at the bottom, the letter "I" in DAPI is green where the rest of the word is blue. Change it to blue."

The scale bars are difficult to see in the top of panel 1F. Also, the tubulin staining in the control panel doesn't look very spindle-like. Do they have a better or higher resolution image?"

We have provided additional images with tubulin staining at different stages of division, these and the IFAs of unexpanded parasites provided in the original figures have been moved to **Supplementary Fig. 3B-C**. Furthermore, we have now performed U-ExM to have better assessment of PfPPM2 localization (**Fig. 1E**) and defects in spindle formation (**Fig. 1F**).

a. It is evident from U-eXM images that PfPPM2 localizes to puncta in schizonts which often coincide with the nucleus or are proximal. In addition, the staining was also seen in the cytoplasm (**Fig. 1E**).

b. U-ExM to localize centrin and tubulin revealed that PfPPM2 depletion results in the loss of spindles as only fragmented tubulin staining was observed, which is in contrast to control parasites that exhibited spindle biogenesis (**Fig. 1F**).

c. We also performed staining with GAP45, which revealed that several parasites did not possess the IMC (**Supplementary Fig. 3E**).

"Fig 2A – explain in the legend what the different colours mean for the proteins highlighted in the graph – in main text described as 3 major families"

We have indicated hypophosphorylated and hyperphosphorylated proteins in blue and red respectively. Further, members of two families of proteins followed in this work are outlined now in cyan and green.

"For 3C, do the authors have any images that would support the graph? Same for 4B"

"We have now provided images of Giemsa smears in these figures"

"Fig 5B, provide scale bar."

Fig 5C – it looks as though the size of the scale bars is written above the scale bars but it is very difficult to see. Remove the writing from the image panels and give the size in the figure legend instead. Also, a statistical comparison could be given between HP1 and S33D for completeness as HP1 is the control"

The scale bar is modified in the figure.

"Fig 5D – statistical comparison could also be given between HP1 and S33D"

The comparison between HP1 and S33D is included.

"Fig 5F is not mentioned in the text"

There is no Fig. 5F.

"Fig 6A – scale bars are very difficult to see and no size is given in the legend. The data in this figure isn't really surprising and doesn't really add much to the story. If S33 has been mutated to alanine, it's no surprise there's no pS33 staining. This could be added to supplementary. Equally, if the key thing here is that S33A mutants have more gametocytes, this message should be clearer in the text rather than the pS33 stain (lines 300-303)."

The endogenous HP1 would still be expressed in WT-HP1 and its S33A /S33D mutants overexpressing parasites. Therefore, pS33-HP1 staining observed in both S33A and S33D parasites reflects phosphorylation of this site on endogenous HP1. Even though both A33 and D33 in these mutants are non-phosphorylatable, it is only in S33A mutant parasites pS33-HP1 staining was not observed and S33D mutants exhibited pS33-HP1 staining. Therefore, S33A mutant overexpression seems to have a "dominant negative" effect on pS33HP1 and induces gametocyte formation which

have reduced pS33-HP1 (**Fig. 6A**). In addition, as suggested by the reviewer, gametocyte formation is accompanied by S33 dephosphorylation (**Fig. 6B**). Therefore, dephosphorylation of S33 seems to promote gametocyte formation and which continues to be almost undetectable in this stage. We have elaborated this in the text (**Page 14, line 305-308, Page 17 373-390**) and hope reviewer agrees that this data can continue to be in the main figure.

Some comments on the text

"Line 32. A more up to date reference is needed here other than Cowman et al. (e.g. Guttery et al, 2022)."

Done

"Line 38 – the statement “the function of most protein phosphatases remains unknown.” Is not strictly true. Guttery et al. 2014 provided evidence of the key functions for many of the P. berghei phosphatases and Morano et al. 2023 showed that the pseudophosphatase PPP8 is required for the maintenance of P. falciparum basal complex integrity. Please correct this."

We have replaced it with "several protein phosphatases remains unknown in *P. falciparum*"

"Line 61 – Please correct (Prommana et al) as no date is given."

Done

"Line 87 and Figure 1F – what do the authors mean by “diffused tubulin staining”? Please clarify this. To my mind, diffused means that tubulin shows staining throughout the parasite, but the figure shows only one tubulin in the + panel of Figure 1F, whereas the graph shows an increase when GlcN is added. This data would be better represented as the number of parasites positive for tubulin staining."

As mentioned above, we have provided U-ExM to demonstrate the defects in tubulin/spindle more effectively (new **Fig 1F**).

We have also provided additional images with tubulin staining at different stages of division on IFAs on unexpanded parasites. We have now provided fresh quantifications for parasites exhibiting normal tubulin staining as suggested by the reviewer. The IFA and quantitation has been moved to Supplementary Fig. **3B,C**.

"Line 143 – the implication that PfPPM2 may negatively regulate PK4 activity is highly speculative. Unless hyperphosphorylation of PK4 can be shown in PfPPM2 depleted cells, I would advise removing this statement."

The quantitative phosphoproteomics data shows that PK4 is hyperphosphorylated (**Supplementary Dataset 1, Fig. 2A, Supp. Fig. S5**), which is why we have speculated that PfPPM2 may regulate its phosphorylation. We have now modified the statement to "PfPPM2 may directly or indirectly regulate PK4" (**page 8, line 148**).

"It is good that the authors have confirmed previous studies showing a decrease in gametocytogenesis in absence of PPM2. Have the authors considered also seeing whether the female:male gametocyte ratio also changes to 1:1, as seen in P. berghei?"

As indicated above, we have not managed to determine the ratio of male and female gametocytes as we do not have specific antibodies against these forms.

"Please check the labelling for genes in the Supplementary RNA-Seq data. Many annotations are wrong. For example, AP2-G (PF3D7_1222600) in the table for NF54 is labelled as "unannotated transcript"."

Our apologies for the oversight and we thank the reviewer for indicating this issue. We have revised the supplementary Dataset 3 to include 'Gene Symbol and Description', providing gene descriptions alongside the gene symbols wherever available. This update ensures accurate and comprehensive annotation of all transcripts.

"Line 331 – Please alter the statement "HP1 at S33 is regulated by PfPPM2". The authors have only shown indirect evidence of this."

Done

"Methods – For qRT-PCR, the authors state that 18S rRNA was used as the HKG, but the supplemental methods state Actin and 18S rRNA was used. Please be consistent. If only 18 rRNA was used, I strongly suggest using an additional HKG as this is common practice."

We agree with the reviewer that using more than one housekeeping gene (HKG) is a good practice. In this study, 18S rRNA was used as the HKG in one set of experiments where sexual conversion was induced by serum depletion (**Fig. 3D**). In other experiments, actin was used as the control.

Since 18S rRNA is widely used as an HKG, we felt it was suitable for normalization in this case. Unfortunately, we do not have leftover cDNA from these experiments to repeat the analysis with actin as a control. However, we would like to highlight that HP1 expression remains unchanged in these experiments, further supporting the reliability of normalization using 18S rRNA.

We hope this addresses the reviewer's concerns.

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3 (Remarks to the Author):

Review of Rawat, et al. NCOMMS-24-59283-T

"The manuscript by Rawat and colleagues evaluates the role of PfPPM2 signaling during the blood stages, asexual and sexual, of Plasmodium falciparum. The study combined genetics, phosphoproteomics, and transcriptomics to demonstrate the multitude of potential functions for PfPPM2. The findings regarding the relationship between PPM2 and HP1 hypophosphorylation are the strongest and most specific finding. The authors provide a series of convincing experiments to demonstrate the importance of this relationship."

We thank the reviewer for appreciating the importance of this study and for providing thoughtful and thorough review of our manuscript.

Major

"1. Figure 1: The description of the localization of PfPPM2 as "present in punctate structures near the parasite nucleus during schizogony, particularly in mid to late schizonts, with some presence in the cytoplasm" is unclear. The immunofluorescence shown in Fig. 1D for schizonts, shows bright punctate staining that is primarily cytoplasmic with fewer, smaller foci of staining that overlap the nuclei. Cross-sectional imaging would help to improve these localization studies. The claim that spindle microtubules are lost in the knockdown is also not well-supported by the images. The +GlcN parasites have multiple nuclei that have divided. Therefore, they must have had spindle microtubules at some point. The associated figure S3D also seems to not match with the phenotype. The staining in S3D shows diffuse localization of IMC1g but normal plasma membrane localization/partitioning with MSP1. The parasites that are formed in the PPM2 knockdown are able to egress and reinvade. This seems like if the IMC were so disrupted that the parasites would not be successful during egress and invasion."

We have addressed these queries by performing Ultra Expansion Microscopy (U-ExM) and providing more images of tubulin staining of unexpanded parasites.

a. It is evident from U-ExM images that PfPPM2 localizes to puncta in schizonts which often coincide with the nucleus or are proximal. In addition, the staining was also seen in the cytoplasm (**Fig. 1E**).

b. We have also provided additional images with tubulin staining at different stages of division these along with fresh quantification are included as new **Supplementary Fig 3B**.

We have now used U-ExM to assess spindle biogenesis, which clearly shows spindle biogenesis in control parasites and images provided in new Fig. 1F reveal the presence of hemispindles, mitotic spindles and interpolar spindles. While most PfPPM2-depleted parasites exhibited fragmented

tubulin staining indicative of loss or lack of spindles, only a very few merozoites/nuclei associated mitotic spindle was observed (**Fig. 1F, Supplementary Fig 3B**). As suggested by the reviewer, PfPPM2 depleted parasites possibly had spindles but were subsequently lost.

c. We also performed staining with GAP45 and analyzed by U-ExM in addition to previously reported IMC1g (**Supplementary Fig. 3D**), which also revealed that several parasites did not possess the IMC (**Supplementary Fig. 3E**). Despite disruption in IMC formation, the lack of defects in invasion of PfPPM2 were surprising. However, recent studies revealed that IMC1g-depleted parasites also do not show significant changes in egress and reinvasion (Cepeda Diaz et al, 2023; Liu et al, 2024). We have mentioned this in the text (**page 6, line 96-99**).

"2. The defects during asexual development seem myriad, including fewer merozoites, abnormal nuclear division, abnormal IMC, decreased protein production, and increased phosphorylation of multiple proteins in multiple processes. It would be helpful for the reader to dissect which of these processes are direct and which are many steps downstream of the PfPPM2 defect. The decreased expression of AP2-G decreases sexual conversion, but it might also increase asexual replication. However, given the widespread defects it is hard to know how this balance plays out."

The major defects upon PfPPM2 depletion are on parasite division and sexual conversion. The formation of fewer merozoites suggested that parasite division was impaired and it may be due to defects in spindle formation, which is critical for division. We also observed defects in protein synthesis most likely a result of aberrant eIF2 α phosphorylation. Although there is no direct evidence in the present study, but protein synthesis is known to play a role in the division of most organisms. It is likely that it is caused by stress on the parasite due to aberrations in some of the other above-mentioned processes. AP2-G is known only to play a role only in sexual differentiation and does not regulate asexual replication (Kafsack et al, 2014).

As suggested by the reviewer, we have summarized some of these possibilities in the **Discussion (page 19, line 421-432)**.

"Minor

1. Line 7 of abstract – “but” should be “by”

2. Line 41, 49, 61, 63, 64 –grammatical errors.

3. Line 64 and Fig 1A are incorrect. Glucosamine does not bind the glmS ribozyme. Glucosamine is converted to glucosamine-6-phosphate, and that is what binds to the ribozyme.

4. Figure 1 seems messy, with differently colored scale bars, some scale bars with lines at their ends, other scale bars without end lines, and font color switches in “DAPI”. This requires some improvement in presentation.

5. Figure 2C has some overlapping text in the AA sequences.

6. Figure 3A has multiple different fonts and some of the AA sequences looks like it is cut off. 3C should also include the raw conversion rates for the gametocyte inductions.

7. Line 242-244 – Why is this a preliminary result? If the result is reproducible, it should not be preliminary. If it is not reproducible, it should be removed from this section. Figure S6A: the top blot needs to be labeled with which antibody is used, presumably anti-HA."

We have made these changes in the revised manuscript.

Reviewer #4 (Remarks to the Author):

"In this study, Rawat et al. describe the role of PfPPM2 during asexual and sexual development in P. falciparum. They further show PfPPM2 removal perturbs the parasite's phosphoproteome leading to increased phosphorylation of several proteins and the authors followed up on Heterochromatin protein 1 (HP1) in detail. They have clearly demonstrated that S33 residue on HP1 is crucial for function and altering to a phosphomutant (A) or phosphomimetic (D) leads to either overcommitment to sexual stages or deleterious effects on asexual growth. Overall, the authors have performed a challenging study and have shed light on the role of phosphorylation on parasite HP1 function. However, to conclude several of their mechanistic hypothesis, additional experiments are required including various experimental controls."

We thank the reviewer for the positive feedback on our study and for highlighting its importance. In the revised manuscript, we have included new controls and performed additional experiments where possible to address the concerns.

Major concerns:

- *"Avoid using green and red together in IFA images and in graphs. They should be recolored for color blind friendly visualization."*

We have made the suggested modifications.

"-Line 70:

a. From the study it appears PPM2 is not essential for completing the asexual life cycle (unless there is some leaky expression from the glmS system) and growth rate is attenuated due to reduced merozoite production. It will be more useful to represent the actual fold change in parasitemia at each cycle and compare to WT instead of normalising WT to 1.0 (Fig 1C). "

Independent studies have indicated that PfPPM2 is refractory to gene disruption (Mamoun & Goldberg, 2001; Mamoun et al, 1998; Zhang et al, 2018), which suggest that it is indispensable for asexual development. Therefore, we used conditional knockdown strategy to study its function. A complete loss in the growth of parasites was not observed in present studies, which as suggested by the reviewer, most likely due to incomplete/conditional depletion of PfPPM2. However, a significant reduction in parasite growth as well as merozoite number was observed, which accounted for impaired growth upon PfPPM2 depletion.

We would like to clarify that we have normalized parasitemia of GlcN-treated parasites - after every 24h - with respect to untreated (-GlcN) ones, which was treated as 1. This allowed us to compare the fold change in parasitemia upon PfPPM2 depletion. Since we have provided actual parasitemia at each time point, which was represented by the line curve, we felt that fold difference in parasitemia at every cycle will allow assessment of direct effect of upon PfPPM2 depletion on parasite growth across 4 replicates. It is clarified in the figure legend.

"Additionally, rather than performing the time course study and classifying the stages as ring/trophs/schizonts (FigS3A), it would be more useful if the number of DAPI/Hoescht stained nuclei were counted over the time course to determine if the reduced merozoite count is due to fewer number of nuclear divisions or a slower cell cycle."

We could have done as suggested by the reviewer but since it was important to assess if the maturation of intraerythrocytic stages was affected upon PfPPM2 depletion, we quantitated each of the developmental stages after microscopic examination, which also allowed us to look at any morphological abnormalities (Supplementary Fig. 2C). If there was a difference in the developmental cycle, it would have been reflected by a delay in development or maturation of Rings>trophozoites>schizonts. Since the distribution of each of these was almost unaltered upon PfPPM2 depletion, delayed development is ruled out. Moreover, we treated parasites with E64 to prevent egress and allow almost complete division and then quantitated the number of merozoites (Fig. 1E). Therefore, the defects in merozoite number are suggestive of impaired parasite division.

"-Give more details in the methods for how invasion rate was determined in PfPPM2-HA-glmS3D7 (SF3C) since the GlcN schizonts make fewer merozoites."

We have provided the protocol for the invasion assay in Supplementary Information, which has been used previously by us and others [e.g.(Rawat et al, 2023)]. In brief, schizonts of untreated and GlcN-treated cultures were incubated with fresh RBCs and the number of rings and schizonts was determined for up to ~10h. Due to egress, schizonts number went down with a corresponding increase in rings, which reflected successful invasion. PfPPM2-depletion did not cause any significant changes in either of the two (modified Supplementary Fig. 2D is provided for more clarity). We had also performed preliminary experiments in which video microscopy was done to assess invasion in real-time at "single-cell" level but did not find any differences. Since we did not have enough replicates, the data was not included as the above-invasion assay was able to address the issue.

"-Line 79 and Fig 2E The observation is confusing and the relevance is not clear : 'PPM2 is present near the nucleus with some presence in the cytoplasm': Near the nucleus would imply localisation in the cytoplasm or another organelle. Higher resolution images (U-ExM)/ super resolution or organellar fraction is required to state if location changes during development.

-Line 86 and Fig1F: IFAs don't have enough resolution to conclude if there is loss of MT spindles. Higher resolution images should be included to justify this statement.

-Line 88 and FigS3D. Presenting diffused staining of IMCg is not sufficient to state formation of IMC is disrupted. Either additional markers of IMC should be presented or TEM should be performed to demonstrate impaired IMC biogenesis."

We have addressed these queries by performing Ultra Expansion Microscopy (U-ExM)

a. It is evident from U-ExM images that PfPPM2 localizes to puncta in schizonts which often coincide with the nucleus or are proximal. In addition, the staining was also seen in the cytoplasm (**Fig. 1E**).

b. U-ExM used to localize and analyze spindle biogenesis revealed that PfPPM2 depletion results in a loss of spindles as only fragmented tubulin staining was generally observed, which is in contrast to control parasites that exhibited proper spindle biogenesis (**Fig. 1F**).

c. We also performed staining with GAP45 and analyzed by U-ExM in addition to previously reported IMC1g , which also revealed that several parasites did not possess the IMC (**Supplementary Fig. 3E**).

"-Line 134 (and Fig S5C): The increased S59 phosphorylation in eIF2alpha is very dramatic. Was this site discovered in phosphoproteomics analysis when PfPPM2 was depleted? If not, please speculate why."

This site was not detected in phosphoproteomics, which could be due to technical reasons like ionization of the peptide bearing this phosphosite, which is critical for mass spectrometry. Given that the phospho-eIF2a antibody is very specific, it served as a good tool to detect phosphorylation of eIF2a as also reported previously (Zhang et al, 2017).

"-Line 139 (and Fig S5D): If protein synthesis is inhibited, the defect on parasite growth should be very pronounced. The quality of blot for the Puromycin incorporation assay is low and the assay should be performed at least two different time points of the parasite life cycle to confirm effect on protein synthesis."

We have performed the puromycin incorporation assay again and the results provided in **Supplementary Fig. 5D**. It is evident that the defects in protein synthesis were observed only at the schizont stage and not at the trophozoite stage, which is probably the reason why drastic defects in parasite growth were not observed earlier in the life cycle.

"-Line 197 pS-33HP1 (Fig 3B) blot is not convincing enough. If there are additional replicates, please show a blot with clearer pS33 band and include a loading control since HP1 levels are different."

We have provided another replicate for the pS33-HP1 blot.

"-Line 228: Reword sentence: 'These observations suggested that PfPPM2 may prevent the association of HP1 and H3K9me3 with heterochromatin...' this suggests PPM2 prevent association of H3K9me3 with heterochromatin which is inaccurate since H3K9me3 is a heterochromatin mark."

We have changed this sentence.

"-Line 228: Since HP1 antibodies are available to the authors (as shown in Fig3B), overexpression of HP1 should be confirmed using antibodies targeting HP1 which would detect both endogenous HP and HP1-GFP tagged protein."

The Western blot was performed using anti-GFP antibody to demonstrate the overexpression of HP1-GFP and is included as **Supplementary Fig. 6A**. We have now performed Western blot with anti-HP1 antibody as well which recognizes both HP1-GFP and the endogenous HP1.

"-Fig4A and B nicely demonstrate that HP1 overexpression lines (although western blots should be performed to confirm this) rescue the PPM2 depletion phenotypes. The authors do not discuss why this might be the case. How does increased levels of HP1 alleviate PPM2 defect?"

We have discussed the reasons why HP1 overexpression reverts defects caused due to PfPPM2 depletion (**pg 17-18, line 374-390**).

"-Line 227: Fig5C does not display Stage IV and V gametocytes. Cells look like Stage III."

We have provided another image which clearly demonstrates Stage IV/V gametocyte.

"-Fig 5E – what stage was experiment performed on? If >50% of the parasites are gametocytes (S33AHP1 line), it is possible HP1 does not bind to H3K9me3 in early stage gametocytes and reduction in S33A-HP1 observed is due to the life cycle stage of the parasites surveyed rather than a property of S33A-HP1. This should be tested by using HP1-GFP line and performing the IP in Stage II/III gametocytes."

We performed the co-IP experiments using HP1-GFP line and compared it with S33A-GFP and S33D-GFP lines. Since these mutants represent hypo and hyper S33-phosphorylated forms of HP1, it was reasonable to use them for assessing interaction of HP1 with H3K9me3 to study the impact of phosphorylation at these sites. As suggested, the S33A mutant parasites are ~50% gametocytes (Fig. 5C) and remaining parasites are possibly sexually committed schizonts and in both cases pS33-HP1 expression is low/absent (Fig. 6A,B). The observations in Fig. 6B suggest that sexual commitment (induced by serum deprivation) results in a loss of pS33-HP1 in both committed schizonts as well as gametocytes. Therefore, it appears that the dephosphorylation of pS33-HP1 may precede gametocyte formation. Therefore, co-IP results with S33A-HP1 mutant may reflect an outcome of HP1-S33 dephosphorylation-mimicked by S33A mutant- and indicate that the loss of phosphorylation of this site may prevent interaction with H3K9me3 as this mutant interacts less with H3K9me3 in comparison to WT or S33D-HP1. As a result, AP2-G expression is induced (Fig. 5D),

which is critical for sexual conversion. It is difficult to obtain enough gametocytes to perform suggested IP experiments but we hope above-mentioned explanation addresses the query.

We have elaborated this in the "Discussion" section (**Page 17-18, line 374-394**).

"- Blots in Fig5E don't look clean enough for accurate proteins quantification (S33D IP and WB , top panel). Moreover, it is important to show input levels of HP1-GFP protein for all lines. This is critical since significant amount of H3K9me3 is pulled down in the NF54 line in the absence HP1-GFP construct (lane1, IP panel)."

Previous studies have demonstrated HP1 and H3K9me3 the interaction in malaria parasite by using recombinant HP1 expressed in bacteria and H3K9me3 peptides (Flueck et al, 2009). Since we had to assess the role of PTM of HP1 (pS33), we had to rely on co-IP performed using parasite lysates to study this interaction. We faced challenges in solubilising HP1, which is tightly bound to chromatin, in a form in which it could be used for H3K9me3 interaction/co-IP studies. For these experiments, the lysates had to be prepared in high salt extraction buffer for IP of HP1 which may contribute to issues in SDS-PAGE/WB. Nevertheless, WBs consistently revealed reduced H3K9me3 in S33A-GFP IP compared to IP from WT or S33D-HP1. We had provided WB using anti-GFP on IP of HP1/S33A/S33D-GFP as the amount of HP1/S33A/S33D-GFP immunoprecipitated in-principle would be utilized for interaction with H3K9me3. It is important to note, as indicated in the figure legend, the amount of H3K9me3 in the IP for three proteins was normalized with respect to the H3K9me3 present in the lysate, which allowed comparison of differences in interaction of H3K9me3-with WT-HP1, S33A and S33D mutants. Since the reviewer suggested, we performed another replicate in which anti-GFP blot on the total lysate was done and is included in the supplement (**Supplementary Fig. 10**), which also revealed similar results (**Fig. 5E**). We hope that the reviewer will agree with our explanation.

"-Line 261 and FigS8B. While it is clear WT HP1-GFP is highly expressed and stabilised in the presence of Shield-1, it is difficult to conclude there is overexpression of S33A in the presence of Shield. The levels of S33A looks similar +/- Shield."

We agree that WT HP1 and its S33A and S33D mutants have different levels of leaky expression- especially for S33A- in the absence of Shield-1 expression which is most likely due to differences in turnover rates/stability of these proteins. Since it is difficult to control this, we have compared their function upon addition of Shld-1.

"-Line 321: The statement PPM2 is dispensible for asexual growth and therefore Pb and Pf PPM2 have diverse function is not accurate. PlasmoGem analysis (<https://plasmogem.umu.se/pbgem/>) demonstrates PbPPM2 ko parasites grow slower which is comparable to the phenotype observed in this work (Fig. 1C)."

We thank the reviewer for pointing this out. We have mentioned this in the manuscript (**page 15, line 326-330**).

"Fig 6. From the FigS8B, it appears the S33A-DD line behaves similarly in the presence or absence of Shield (protein levels and growth rate). Therefore, the rationale of using Shield in the experiments shown in Fig6A is not clear."

As mentioned above, WT HP1 and its S33A and S33D mutants have different levels of "leaky" expression in the absence of Shield-1 (Supplementary Fig. 8B), which is most likely due to differences in turnover rates/stability of these proteins. Also, there is a possibility of experiment-to-experiment variation when it comes to "leaky" expression. Therefore, in order to stabilize the expression of the three proteins, the experiments were performed in the presence of Shield-1. Since Shld-1-treatment results in "almost" similar and higher expression levels of the two mutants (Supplementary Fig. 8B), the experiments in Fig. 6A directed at assessing pS33-HP1 were performed under these conditions.

Minor comments:

"Fig 2C – add error bars to growth curves. Indicate on graph when GlcN was added."

Done

"Lines 168-184: Since the authors performed extensive analysis of RNA, proteome and phosphoproteome it would be useful if the authors presented /discussed effects of PPM2 are specifically on the phosphoproteome (without affecting protein levels) and which changes are due to differences in the proteome."

We performed simultaneous analysis of the proteome and phosphoproteome of each sample. Using these datasets, we normalized the alterations of phosphorylation events against the expression of proteins. Therefore, the changes in phosphorylation discussed in the manuscript are independent of the changes in levels of corresponding proteins. We have elaborated this in the methods section in the supplement.

"Fig 3E : number of replicates and error bars should be included."

We had provided a representative of three independent replicates. We have performed one more replicate and provided the SEM+/- SE and included in the figure.

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Reviewer #1 (Remarks to the Author):

"The authors have addressed my comments and provided expansion microscopy images for tubulin and centrin. This is more clearer in the present manuscript. They have scored the tubulin signal in the mutant versus control but it would have been good to have centrin the same in Fig1F to have complete quantification.

It is appreciated that authors have provided additional images at different stages and also performed U-ExM, please could the authors clearly label which stage of schizont development is which in Figure 1E since they mention "different stages of development". Please be clear what timepoint each row is. Please also make this clear in the text and methods."

"Overall most comment were addressed and provided valid discussion."

We thank the reviewer for appreciating our effort to address the queries satisfactorily.

U-ExM was done during early-mid schizont development, which corresponds to ~36-44hpi. We have indicated this in the figure legend. We had performed quantification of centrin foci on unexpanded parasites as it was easier to do this on images of unexpanded parasites and have provided the quantification in Supp. Fig. S3C along with images from IFA on unexpanded parasites.

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3 (Remarks to the Author):

Review of Rawat, et al. NCOMMS-24-59283-T

"The revised manuscript by Rawat addresses some of the concerns from the previous reviews. As noted previously, their findings regarding the relationship between PPM2 and HP1 hypophosphorylation are the strongest and most specific finding. The microscopy data to support their conclusions remains somewhat weaker than the proteomic and more functional studies. This is a minor aspect of the study, so this weakness is less important. The authors continue to provide a series of convincing experiments to demonstrate the importance of the relationship between PPM2 and HP1. "

We thank the reviewer for appreciating the changes in the manuscript.

Major

"1. Figure 1: The authors should be commended for their use of U-ExM. This is a major improvement from the images in the first submission. While the localization claims are not critical for the study, they could be displayed more clearly in the figure. It looks like the U-ExM images are maximum projections, which really is not the correct method to show localization or, more specifically, to show co-localization. The authors utilized z-sections to obtain the data, but the axial localization differences are lost in maximum projections. In addition, all images should be treated with the same settings – laser power, gain, and post-processing. This allows the reader to know the relative amount of the protein present. For example, the dim staining may just be background given that a negative control (untagged parasite) is not shown for the mouse anti-HA antibody."

MIP, which is commonly used for U-ExM image analysis, allowed the illustration of the global localization of PfPPM2, which is present both in the cytoplasm and is proximal to the nucleus. We agree with the reviewer that z-sections are better for assessing co-localization. Therefore, we have now provided 3-D projections of the Z-sections for two of the images as accompanying videos (V1 and V2). We had increased the brightness of one of the images and indicated it in the legend only for the purpose of illustration but have now reverted to the original as suggested by the reviewer.

"In 1F, the graph is a nice attempt to provide some quantification of the phenotype. However, the meaning of "parasites with normal tubulin staining" is unclear. In order for this quantification to be meaningful, highly synchronized parasites with the same number of nuclei should be compared. The meaning of the graph is lost somewhat without more precise clarification. Also, are these parasites from the first cycle after knockdown or the second?"

The parasites/schizonts used in the reported studies are from cycle 1. We have compared parasites with a largely similar number of nuclei for quantification. Parasites exhibiting hemispindles, mitotic or interpolar spindles as shown in -GlcN (untreated) parasites were considered as "parasites with normal tubulin staining", which we have clarified in the legend.

"2. Sup Fig 2D – The statement that there was no difference in invasion is a little hard to understand. If fewer merozoites are formed then how is the rate of invasion the same for knockdown parasites. The decreased number of merozoites is likely the source of the replication defect shown in fig 1. I guess that the sup fig 2d is the first cycle, and the replication defect is not fully realized until the second cycle. If there were no difference in the number of new rings formed, then there would be no growth defect. This should be clarified. Perhaps the best thing would be to show the relative invasion for the second cycle instead. Lines 65-71 are somewhat confusing as currently written. "

The author is correct that the growth defect observed is indeed due to a smaller number of merozoites formed. As a result, the number of rings formed in the next cycle is lesser in +GlcN parasites. We have clarified this further in the manuscript on page 5, lines 66-74. However, to assess if invasion was impacted, independent re-invasion assays had to be performed. Since most of the experiments that reflected defects (smaller number of merozoites formed, defects in spindle

formation etc) these assays were done with cycle 1 schizonts (~80-90hpi), and the invasion assays were also performed using cycle 1 schizonts.

The invasion assay protocol could possibly explain the reason for the lack of differences in rings formed: It has been observed that in invasion assays, reported here and previously published, only ~10-20% merozoites successfully invade RBCs and there are always excess (~60%) merozoites that do not invade, which could be due to *in vitro* culture conditions. Therefore, even if there is a decrease in the number of merozoites, which was the case in PfPPM2-depleted parasites that had ~40% fewer merozoites, it may not result in reduced ring formation. On the contrary, if this population of "active" merozoites both in control and knockdown conditions, does not have defects in ability to invade, it would not result in a decreased ring formation, which is the case in the present study.

"3. Sup Fig 3D and 3E. I mentioned this in the previous section. While I appreciate the comments from the authors, it does not make sense that invasion is "normal" when both IMC1g and GAP45 staining are so abnormal. The manuscript that is mentioned in the rebuttal letter about IMC1g shows relatively normal GAP45 staining during segmentation (see Fig 2E in Cepeda Diaz mBio 2024). Again, I think that this is not the major focus of the study. However, the data of abnormal GAP45 and normal invasion are not entirely consistent. I am not sure that this needs to be reconciled, but it is worth a bit more description of this putative inconsistency in the text than is currently present. "

In the mentioned study by Cepeda Diaz et al, U-ExM was not performed for GAP45 localization, which is probably why defects do not look as severe as evident from the present work. As reported in Supp. Fig S3D/E, ~50% of schizonts exhibited defects in IMC1g and GAP45. Further, not all merozoites in these schizonts exhibited abnormal GAP45/IMC1g staining (additional images included in Supp. Fig. S3E). Therefore, it is possible that there were enough number of invasion-competent merozoites with intact IMC/glideosome, which resulted in no significant defects in invasion upon PfPPM2-depletion. We have tried to explain these points on page 6, line 104-109.

"Minor

1. Line 38 – as noted by one of the other reviewers, this statement should be modified to reflect the data from the P. berghei study. The loss of PbPPM2 caused a "significantly slow" growth phenotype. The current version suggest that there was no growth defect and should be clarified to aid the reader's understanding.

2. Line 75 – the term Ultra Expansion Microscopy is incorrect."

We have made these corrections

Reviewer #4 (Remarks to the Author):

"The authors have performed additional experiments and addressed majority of my concerns. However, a few matters require clarification before publication:

1.Fig1E: mention which lifecycle stages are examined (hpi?) PfPPM2 has an interesting staining pattern and could be localised at the MTOC in late-stage parasites (bottom 2 rows). The authors should provide 'zoomed' images of PPM2 localisation in these parasites. This could explain the fragmented tubulin staining in phosphatase depleted mutants in Fig 1F"

U-ExM was performed on schizonts during various stages of division (~36-44 h.p.i). We have provided zoomed images of individual merozoites in the figure. In addition, we have also provided videos of projection of z-stacks to further highlight the localization of PfPPM2.

"2.Fig1F: mention how many parasites were counted."

~65 parasites were counted, indicated in the legend.

3.FigS6A: The additional western blot with HP1 antibody shows a very minor increase HP1 levels in the over expressing line (compare endogenous HP1 level 35kDa to HP1-GFP at 60kDa). The authors should discuss whether such a small increase in HP1 protein levels actually rescues the phenotype or the OE plasmid might change HP1 properties whether it is regulation, stability etc.

The anti-GFP blot shows that HP1-GFP is significantly overexpressed. It is possible that the anti-HP1 antibody did not recognize HP1-GFP proteins with similar efficiency as the untagged endogenous HP1. The data shows that the amount of HP1 overexpressed was able to revert growth defects. One of the possibilities is that the crt-promoter, which was used for HP1- overexpression facilitated more sustained expression of HP1 during parasite development, which contributed to these effects. We have mentioned this on page 12, line 255-257.

"4.Although the authors claim that taken together their pulldown data in Fig 5E and immunofluorescence in Fig6A give evidence HP1-S33A-GFP is does not bind H3k9me3, this evidence is circumstantial since the pull down is performed in different developmental stages. If it is challenging to perform the pulldown experiments in stage II gametocytes, at least this caveat should be discussed."

We have mentioned this in the text page 18, line 405-409.

"Minor comments:

1. Maintain consistency in error bars. Some graphs have bi-directional and others are top (even when showing the same assays e.g Fig1C vs Fig4A
- 2.This could be due pdf conversions, but will be useful to check space between panels in IFA images.
- 3.Correct annotation of gene- either use AP2G or ap2g in Fig6C"

We have made these corrections in the revised manuscript.