EMBO reports

# Phosphorylation of myosin A regulates gliding motility and is essential for Plasmodium transmission

Johanna Ripp, Xanthoula Smyrnakou, Marie-Therese Neuhoff, Franziska Hentzschel and Friedrich Frischknecht
**DOI: 10.15252/embr.202254857**

*Corresponding author(s): Friedrich Frischknecht (freddy.frischknecht@med.uni-heidelberg.de)*


## Review Timeline:

| | |
|---|---|
| Transfer from Review Commons: | 14th Feb 22 |
| Editorial Decision: | 14th Mar 22 |
| Revision Received: | 18th Mar 22 |
| Editorial Decision: | 29th Mar 22 |
| Revision Received: | 6th Apr 22 |
| Accepted: | 8th Apr 22 |

Review COMMONS

*Editor: Achim Breiling*


## Transaction Report: This manuscript was transferred to EMBO Reports following peer review at Review Commons

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

# Revision 0

# 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)

Less than 1 month

## 2. Evidence, reproducibility and clarity:

### Evidence, reproducibility and clarity (Required)

**Summary:**

Malaria parasites undergo a complex life cycle including invasion of multiple cell types in mosquito and human hosts, each presenting different mechanical challenges. The parasite uses a myosin motor, MyoA, for force generation and there is evidence that phosphorylation at the N-terminus regulates motor properties, but the role of MyoA and its regulation have not been directly studied in the fastest moving stage, sporozoites. Using a rodent malaria model to enable investigation of the entire parasite life cycle, the authors generated several mutations in the N-terminus of MyoA, including S19A, designed to test motor properties and phospho-regulation.

The authors found that standard genetic modification techniques interfere with myoA expression, so had to employ a different, marker-free approach. These mutants showed only mild changes in fitness until sporozoites invade the salivary glands, where MyoA-S19A parasites, but not other mutants, were strongly impaired, revealing a significant mechanical barrier. Tracking isolated sporozoites showed that motility of mutant sporozoites, especially MyoA-S19A, is significantly reduced, and using an optical tweezer system the authors directly measured a reduced force generation capacity for some of the mutants. Together, these findings show the importance of MyoA, and specifically phosphorylation of S19, for sporozoite motility, and point to invasion of the salivary glands as a key mechanical challenge that the acto-myosin machinery may be optimised to overcome.

**Major comments:**

The data is clear, and results are well presented and analysed. However, some of the interpretation should be qualified or discussed further, given what has been shown previously about these mutants.

The claim that the salivary glands represent the strongest obstacle to parasite transmission rests primarily on the phenotype of the S19A mutant, which shows a striking failure to invade salivary glands and the strongest defects in motility of isolated sporozoites but is not strongly affected in other stages.

However, this result is also consistent with an alternative hypothesis (mentioned in the introduction) proposed from structural and biochemical data from Plasmodium falciparum, which predicted that the S19A mutation would not reduce the overall force production capacity of MyoA, but would specifically impair fast gliding sporozoites. The loss of phosphorylation in this mutant was proposed to fix the motor in a high force/low speed state (Robert-Paganin et al, 2019). This is also consistent with the data here showing the phosphomimic S19D mutant was specifically impaired in blood stages and not salivary gland invasion, albeit with a much lesser degree of impairment.

The authors imply that the S19A mutation gives the strongest perturbation of MyoA force generation capacity (e.g. line 305, line 714) based on this mutant having the strongest defects in sporozoite velocity, but unfortunately it was not possible to directly measure the force production capacity of the S19A parasites. The authors do show that the E6R mutant (which behaved similarly to S19A in biochemical experiments) had lower sporozoite force capacity. However, without the S19A force production data or other mutants impaired in salivary gland invasion, it is premature to dismiss the alternative explanation that the S19A mutation alters MyoA regulation, rather than its overall force generation capacity. This mutation would lead to a high force/low speed motor specifically impaired in sporozoite motility, rather than salivary gland invasion being the greatest obstacle overall.

Therefore, the authors should discuss how their data compares to existing biochemical data on these mutants (e.g. in vitro MyoA-E6R motors showed increased force production, in contrast to the sporozoite data shown here) and justify further how their mutants show that salivary glands are the strongest barrier to transmission, overall.

**Minor comments:**

The figures are clear and prior studies are referenced appropriately.

Some minor points that the authors may want to clarify or discuss:

The data shown in Fig 3G shows that S19A mutant parasites have a one-day delay/90% reduction in mouse infectivity, however given the striking decrease in salivary gland sporozoite numbers, a lower level of infection might be expected. The much lesser defect in Fig 3H suggests that most of the reduction in infectivity was due to reduced sporozoite numbers during natural transmission (or a defect in skin migration). The authors present this clearly in the discussion but may want to make this clearer in the results section.

Regarding the successful cloning strategy, the authors may want to clarify why they chose the ama1 promoter for their constructs, and discuss whether any of the small differences between WT parasites and WT recon (e.g. reduced hemolymph sporozoite speed) are important.

On line 669 (Fig 3 legend), it says "the N-terminal extension is depleted", instead of "deleted".

# 3. Significance:

## Significance (Required)

Recent studies working at the biochemical level or with the blood stages of the parasite life cycle have started to investigate the role and regulation of the MyoA N-terminal extension and S19 in parasite motility. However, this study was able to provide a holistic understanding of multiple motor mutations across the entire life cycle using the rodent malaria model, something that is extremely difficult to do with human

malaria parasites. This was complemented by a broad characterisation of motor mutants, using live cell microscopy tracking in 3D, optical tweezers and standard growth assays to identify a specific growth defect for the S19A mutant in invading salivary glands. This is one of the first pieces of direct evidence for a critical role of MyoA in sporozoites.

This work will be of keen interest for those in the field of motility and invasion of the malaria parasite and related parasites, as well as showing technical advances useful for those interested in genetic modification or biophysical analysis of Plasmodium berghei parasites. Finally, the conclusions of the study hint at implications for the evolution of Plasmodium and other arthropod parasites.

# 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)

Ripp et al analyze the effects of preventing phosphorylation of Ser19 of P. berghei MyoA on the parasite's ability to invade different cell types encountered in its life-cycle. MyoA is a critical component of the parasite's actin-myosin motor that is required in the mammalian host for invading hepatocytes and RBCs , in the mosquito for invading midgut and salivary glands. Each of these invasions engages the acto-myosin motor. Motor-driven motility is a key feature of the invasion steps. Here, the authors focus on motility of hemolymph and salivary gland sporozoites. Since X-crystallographic evidence implicates two amino acids of MyoA (E6 and phosphorylated S19) in MyoA kinetics, authors test the functional relevance of these two amino acids on motility by engineering point mutations in E6 and S9. E6 is substituted and the importance of S9's phosphorylation is interrogated by generating a phosphomimetic mutant (S19D) to mimic constitutive phosphorylation and an unphosphorylatable mutant (S19A). The mutants were taken through the entire lifecycle.

Data demonstrate that S6 mutations decrease the speed but not the fraction of motile sporozoites that is motile. This is interpreted to mean the S6 phosphorylation status is important for force generation. Force generation is assayed directly through an elegant bead-pulling assay.

The manuscript is generally well written and results are clearly described. There should be clearer explanation for the specific focus of E6 and S19.

**Major comments**

1) ookinete motility should be determined. Since the MyoA-based motor is required for ookinete movement, the effect of E6 and S19 mutations on ookinete motility should be established. Oocyst numbers are only an indirect measure and their being normal cannot necessarily be assumed to imply that ookinete motility is unaffected.

2) The delay in patency of S19A sporozoites is attributed, in part, to defective hepatocyte invasion in vivo. In vitro data suggest that sporozoites movement through the skin will be reduced. Authors should determine sporozoite invasion rate and infectivity in vitro to obtain a more direct measure of the S19A substitution's effect on invasion and consequently infection.

3) Data charts do not contain any error bars. This should be rectified. Fig 4A should have p-value since the difference in % motile hemolymph sporozoites appear significant.

4) Gene-targeting figures in Supplementary Material (Supp Fig. 1A and Fig. 2A and 2B) are difficult to follow as cloning strategies are totally unclear. Without significantly more detail, it is impossible to understand how mutant lines were generated. One example, in Supp Fig 2A how was the pbMyoA5'ama recipient line produced and how are mutations introduced into it? Does negative selection lead to excision of the marker? Is there a homologous recombination event, if so between which sequences? In Supp Fig 2C why is an amplicon absent in 'Sel' lanes of WT reconstitution, E6R, S19A, S19D but present in Recipient line? Supp Fig 2B appears to show a deletion is the C-terminus of MyoA deltaN mutant.

Methods description of gel migration assays and force measurements should be clearer.

5) Amount of MyoA protein in all mutants and reconstituted WT need to be established. Observed phenotypes could be due, in part, to reduced protein. Related, is there evidence for altered MyoA phosphorylation in the S19 mutants? Alternative phosphorylations in vivo can occur and it would be interesting to know this.

6) In fig 3C growth rate of S19D should be compared with the WT recon not RL.

7) Increased hemolymph/middgut ration of S19A is quite interesting. Authors state "motility is required for egress from oocysts". These data appear to contradict this statement. Is there an implication that MyoA phosphorylation inhibits exit of sporozoites from oocysts, or that a different Myosin is required for egress than invasion?

# 3. Significance:

## Significance (Required)

The manuscript represents an advance in our understanding of the in vivo role of MyoA phosphorylation. Authors made creative use of MyoA mutagenesis to address the in vivo functions of specific amino acids fingered in vitro as playing an important role in MyoA dynamics. Their focus on salivary gland invasion and hepatocyte invasion is significant since these invasive steps are critical for the parasite's life-cycle. Some conclusions need to be shored up through more direct experimentation in an otherwise robust manuscript.

# 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)

This manuscript examines the importance of a key myosin phosphorylation site in the regulation of parasite motility, throughout the lifecycle of malaria parasites. Previous in vitro work demonstrated that phosphorylation of S19 increases Myosin stroke speed at the expense of force. Subsequent work in P. falciparum also demonstrated that mutations impairing the effect of S19 mutations reduced merozoite invasion efficiency. Here, generate a selection of mutant transgenic parasites including S19A and phosphomimetic S19D and demonstrate that interference with phosphorylation at this site affects asexual growth, sporozoite invasion in mosquito and mouse, but interesting with no apparent effect on ookinete traversal of midgut wall. Of these effects, the most dramatic was a reduction in ability of S19A mutants to invade salivary glands, thus highlight an important role for myosin phosphorylation/tuning during this stage. Interesting a detailed analysis of both sporozoite speed, force generation and motility characteristics, demonstrate that both S19A and S19D reduce motility speed and S19D reduces force. Not only does the work succeed in characterise the role of Myosin tuning throughout the lifecycle for the first time, but also represents the first detailed attempt to dissect the linkage between the in vitro Myosin tuning state with the actual parasite speed and force. As such the work reveals a far more complex role for Myosin tuning than previously hypothesised and marks major step forward in our understanding of how this works across the lifecycle of malaria parasites.

The experimental data are extremely robust throughout and the paper offers a sophisticated analysis of key motility parameters and force generation. The majority of my comments are minor, and no additional experimental work is required. My only negatives with this manuscript is that there are key areas where the novelty of the work here is undersold - most notably that this is first time parasite motility and force has been examined in relation to tuning state. This seems to be based on the assumption that fast myosin will definitely make fast parasites rather than a more nuance use of this in the control of parasites. This is not the case and the data presented here generally points to the importance of dynamic Myosin tuning rather than one or other state being preferrable for a given invasive stage. That is fascinating and quite distinct to previously proposed roles for tuning. Finally the only other major issue I have is with the statements regarding the relative importance of salivary gland invasion in evolution of the parasite. I explain more in comments below, but I really feel this should be removed as it is both speculative and distracting.

**Major**

• The experimental approaches are all robust, have adequate replication and statistical analysis and includes both classic phenotypic analysis of mutant parasites through transmission with some very elegant detailed analysis of both sporozoite force, speed and motility parameters.

• L251, L335 and abstract, Authors Identify the main barrier for sporozoites unable to phosphorylate S19 as being salivary gland entry and therefore this is the main obstacle against which malaria parasites must evolve their migration and invasion machinery. This sentence (and equivalent in significance statement) come across as a big and leap, particularly against the really solid work that leads to it. Whilst I think I follow the logic, I see this as quite unnecessary and speculative. I strongly feel the authors should change it. The invasion machinery evolved well before the malaria sporozoite (even if early hosts were insects) and there are likely numerous specialist adaptations and posttranslational modification vital for each transitional step. Instead the authors should consider focusing on how their data has demonstrate differential importance for S19 regulation across the lifecycle, and that regulation of MyoA force and speed clearly does not correlate directly with speed and force of parasite. This highlights the complexity of parasite motility and the need for complementary in vitro and in vivo (or in parasite) experiments.

• L269 I think it is important to note that Blake et al demonstrates that an equivalent of the S19A mutation (K764E) causes an impairment of progression between deformation and invasion. The models to explain this using force thresholds, based upon the findings in Robert-Paganin et al., whilst compelling are theoretical, and the links for parasite force generation and speed are actually only tested here for the first time. Its important that this previous gap in our knowledge is recognised and the important efforts to characterise the effect on parasite speed and force made here. The work here goes quite significantly beyond characterisation possible in merozoites, where levels of deformation were the main characteristic measured.

• L283 I think the discussion oversimplifies in several places, and in doing so skims over some of the most interesting findings. I think its important to recognise that the "fast myosin" is probably dynamically required for particular steps rather than S19P simply meaning faster sporozoites. Data also showed that the D mutant also results in slower sporozoite motility - so its actually the dynamic regulation that appears to be important for speed. In addition specific stages appear more sensitive to S19A than S19D suggesting being locked in S19P is less detrimental than locked in S19. Sentences like L283 here could be modified to better reflect this.

**Minor**

Figure 2 clarify whether 2 independent cage experiments were carried out on separate days with initiated with independent passages.

Fig 2e legend indicates statistical analysis were carried out on E, but there are no indicators as to result in figure.
L166 The comparison with wt/S19A is interesting as it appears there is a single outlier reading with high growth rate. It seems likely that there is a difference here but with insufficient power to distinguish it (in part by need to adjust for multiple comparison). It's a tricky one, but wording of the S19A growing as well as wt, could perhaps be changed to the more technical "was not significantly different to wt"? On a wider point these slow growers are interesting as they would likely be very susceptible to adaptations. Could the authors note how passage numbers were regulated for these lines (i.e. to avoid experiments with high passages, I am sure they already were for gam formation).

L192 Can the author back calculate the likely difference in parasite number resulting in a 1 day delay? This will help contextualise the effect of delayed parasitaemia (e.g. XX days delay is equivalent to XX lower

hepatic schizonts). Was any statistical analysis undertaken to look at this delay?

261 "Although invasion of the red cells appears as the easiest of these tasks for the parasite 261 due to the short time and distance," I think this needs rewording. I don't think we can make assessments of "Easy" because it must happen quickly. Perhaps more appropriate to separate out discussion of migration and invasion. The former is clearly more straight forward for meros, but invasion likely is highly complex for both meros and sporozoites (and simpler for Ookinetes).
L292 Worth noting S19 was also identified as being differentially phosphorylated across compound 2 (PKG inhibitor) treated schizont lines https://www.nature.com/articles/ncomms8285 - again supporting role for cGMP/Ca2+ regulation.
L325 This was a very interesting experiment, worth expanding on why this swap is interesting and how it could be used. Highlight that MyoA forms complex comment on whether Pb lightchains likely to still bind.

# 3. Significance:

## Significance (Required)

The key conceptual advance of this work is in extending our knowledge of the role of myosin tuning in motility and invasion of parasites, particularly those within the Apicomplexan phylum. Building on the finding that myosin phosphorylation can increases motor speed in vitro, and blocking this impairs P. falciparum merozoite invasion, the work examines this throughout the lifecycle. The most exciting findings are the relatively differential importance at different lifecycle stages- with a large impact during salivary gland invasion and no effect on midgut traversal. Finally the work to link tuning state to specific parasite motility characteristic is a novel step and suggests tuning plays a complex role that dose not just simply increase motility speed.

These findings will be of significant interest to those looking at the relationship between motor protein regulation and cellular phenotypes, host pathogen interactions and particularly those working on invasion and motility of apicomplexan parasites.

The work also provides some interesting technical innovation through its exploration of approaches to modify the MyoA locus. The demonstration that generic 3'UTR function poorly during transmission stages is an important finding and will be of significant interest to anyone undertaking genetic modification of parasite.

I have worked on motility and invasion in both rodent and human malaria parasites, predominantly on ookinetes and merozoites. This has included significant work on the studying regulation of the parasite motor complex in P. falciparum and more recently work on gliding and motility in P. knowlesi merozoites.

All the best,
Rob Moon

**Referee Cross-commenting**

I agree with reviewer 2 point regarding ookinete motility. It would be interesting to assess this. Measurements would be quite a significant amount of additional work so as alternative, it would be important to at least note that if motility is impacted in ookinetes, it is not sufficient so as to reduce ookinete-> oocyst transition.

My interpretation of the final constructs is that mutant MyoA being placed under endogenous MyoA promoter, not AMA1 promoter as described by Reviewer 1. I had thought same on first read, and reviewer 3

also raises comments about difficulty in understanding cloning screen. I think the authors need to improve how this complex strategy is explained.

Dear Ms Monaco, dear colleagues,

Thanks for returning three constructive reviews on our paper. Please find below our answers to their comments. We took the time to conduct the two suggested experiments (done by Dr Franziska Hentzschel, who is now a new co-author). We are grateful to the reviewers for suggesting these as one yielded an indeed interesting new insight concerning ookinete motility, which we have added as part of figure 3: the mutants differentially affect ookinete motility with E6R and S19A slowing it down, while surprisingly, S19D ookinetes moved faster. We think that the molecular insight into the function of myosin across the life cycle will be interesting for readers of *EMBO reports*.

Sincerely,

---

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

**Summary:**

Malaria parasites undergo a complex life cycle including invasion of multiple cell types in mosquito and human hosts, each presenting different mechanical challenges. The parasite uses a myosin motor, MyoA, for force generation and there is evidence that phosphorylation at the N-terminus regulates motor properties, but the role of MyoA and its regulation have not been directly studied in the fastest moving stage, sporozoites. Using a rodent malaria model to enable investigation of the entire parasite life cycle, the authors generated several mutations in the N-terminus of MyoA, including S19A, designed to test motor properties and phospho-regulation.

The authors found that standard genetic modification techniques interfere with myoA expression, so had to employ a different, marker-free approach. These mutants showed only mild changes in fitness until sporozoites invade the salivary glands, where MyoA-S19A parasites, but not other mutants, were strongly impaired, revealing a significant mechanical barrier. Tracking isolated sporozoites showed that motility of mutant sporozoites, especially MyoA-S19A, is significantly reduced, and using an optical tweezer system the authors directly measured a reduced force generation capacity for some of the mutants. Together, these findings show the importance of MyoA, and specifically phosphorylation of S19, for sporozoite motility, and point to invasion of the salivary glands as a key mechanical challenge that the acto-myosin machinery may be optimised to overcome.

Response: Thanks for this concise and precise summary of our work and for recognizing the massive amount of work that went into showing that ‘standard approaches’ did not help us in the first place. We do hope that also this part will be important for some colleagues in the field.

**Major comments:**

The data is clear, and results are well presented and analysed. However, some of the interpretation should be qualified or discussed further, given what has been shown previously about these mutants.

The claim that the salivary glands represent the strongest obstacle to parasite transmission rests primarily on the phenotype of the S19A mutant, which shows a striking failure to invade salivary glands and the strongest defects in motility of isolated sporozoites but is not strongly affected in other stages.

However, this result is also consistent with an alternative hypothesis (mentioned in the introduction) proposed from structural and biochemical data from Plasmodium falciparum, which predicted that the S19A mutation would not reduce the overall force production capacity of MyoA, but would specifically impair fast gliding sporozoites. The loss of phosphorylation in this mutant was proposed to fix the motor in a high force/low speed state (Robert-Paganin et al, 2019). This is also consistent with the data here showing the phosphomimic S19D mutant was specifically impaired in blood stages and not salivary gland invasion, albeit with a much lesser degree of impairment.

The authors imply that the S19A mutation gives the strongest perturbation of MyoA force generation capacity (e.g. line 305, line 714) based on this mutant having the strongest defects in sporozoite velocity, but unfortunately it was not possible to directly measure the force production capacity of the S19A parasites. The authors do show that the E6R mutant (which behaved similarly to S19A in biochemical experiments) had lower sporozoite force capacity. However, without the S19A force production data or other mutants impaired in salivary gland invasion, it is premature to dismiss the alternative explanation that the S19A mutation alters MyoA regulation, rather than its overall force generation capacity. This mutation would lead to a high force/low speed motor specifically impaired in sporozoite motility, rather than salivary gland invasion being the greatest obstacle overall.

Therefore, the authors should discuss how their data compares to existing biochemical data on these mutants (e.g. in vitro MyoA-E6R motors showed increased force production, in contrast to the sporozoite data shown here) and justify further how their mutants show that salivary glands are the strongest barrier to transmission, overall.

A: thanks for these critical but very constructive comments. We agree that the S19A-force linkage is too much of a conjecture and toned down the statement concerning the primary importance of salivary gland invasion. We also agree (and actually actively pursue) that in in vitro findings often don't translate into similar in vivo finding, e.g. if a motor produces more force in vitro does not mean it also does in vivo, where multiple effects might outweigh or even cancel each other.
We have now changed the text at several occasions to take these comments into account, e.g. the second part of the abstract now reads "In contrast to data from purified proteins, both E6R and S19D mutations lowered force generation by sporozoites. Our data show that the phosphorylation cycle of S19 influences parasite migration and force generation and is critical for optimal migration of parasites during transmission from and to the mosquito." (lines 38-39).
Also, in the discussion we added the following paragraph: "Interestingly, the E6R mutation caused an increase in force production *in vitro* (Moussaoui et al, 2020).

While these results appear contradictory, they might not be, as an increase in force on a single molecule level does not necessarily have to scale to an observable increase in force on the cellular level, where multiple myosins and actin filaments come together to generate an ensemble force, which is measured in our assay (Brown +Bridgman 2003; Quadt et al, 2016). The E6R mutation also caused a reduced speed of moving actin filaments, which might *in vivo* be correlated with a lower force." (lines 366-372).

**Minor comments:**

The figures are clear and prior studies are referenced appropriately.

Some minor points that the authors may want to clarify or discuss:

The data shown in Fig 3G shows that S19A mutant parasites have a one-day delay/90% reduction in mouse infectivity, however given the striking decrease in salivary gland sporozoite numbers, a lower level of infection might be expected. The much lesser defect in Fig 3H suggests that most of the reduction in infectivity was due to reduced sporozoite numbers during natural transmission (or a defect in skin migration). The authors present this clearly in the discussion but may want to make this clearer in the results section.

A: we now added a statement reading "This suggests that indeed the most important contribution to diminished transmission is the lower number of parasites present in the salivary gland." to the results, lines 255-257. Note that due to the inclusion of the ookinete data, this now refers to Figure 4C,D.

Regarding the successful cloning strategy, the authors may want to clarify why they chose the ama1 promoter for their constructs, and discuss whether any of the small differences between WT parasites and WT recon (e.g. reduced hemolymph sporozoite speed) are important.

A: thanks, we chose the ama1 promoter as this was used before by colleagues. This is now stated clearer in the text; lines 169-171: "This choice was motivated by the prior successful promoter swap, where the myoA promoter was changed to the ama1 promoter and shown to arrest ookinete motility (Siden-Kiamos et al., 2011)." Note that in the final myoA mutant lines, myoA expression is again driven by the endogenous myoA promoter.

We also added a line concerning the small differences in the discussion (lines 454-458: "We also noted small differences between the reconstituted WT parasites and WT parasites in the speed of hemolymph sporozoites and salivary gland derived sporozoites migrating in 3D (but in no other parameter), which could potentially be attributed to the fact that the reconstituted WT parasites are clonal lines or to experimental variation.").

On line 669 (Fig 3 legend), it says "the N-terminal extension is depleted", instead of "deleted".

A: corrected (legend to Figure 3B)

Reviewer #1 (Significance (Required)):

Recent studies working at the biochemical level or with the blood stages of the parasite life cycle have started to investigate the role and regulation of the MyoA N-terminal extension and S19 in parasite motility. However, this study was able to provide a holistic understanding of multiple motor mutations across the entire life cycle using the rodent malaria model, something that is extremely difficult to do with human malaria parasites. This was complemented by a broad characterisation of motor mutants, using live cell microscopy tracking in 3D, optical tweezers and standard growth assays to identify a specific growth defect for the S19A mutant in invading salivary glands. This is one of the first pieces of direct evidence for a critical role of MyoA in sporozoites.

This work will be of keen interest for those in the field of motility and invasion of the malaria parasite and related parasites, as well as showing technical advances useful for those interested in genetic modification or biophysical analysis of Plasmodium berghei parasites. Finally, the conclusions of the study hint at implications for the evolution of Plasmodium and other arthropod parasites.

A: thanks for appreciating the value of our study for a broad audience.

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

Ripp et al analyze the effects of preventing phosphorylation of Ser19 of P. berghei MyoA on the parasite's ability to invade different cell types encountered in its life-cycle. MyoA is a critical component of the parasite's actin-myosin motor that is required in the mammalian host for invading hepatocytes and RBCs , in the mosquito for invading midgut and salivary glands. Each of these invasions engages the acto-myosin motor. Motor-driven motility is a key feature of the invasion steps. Here, the authors focus on motility of hemolymph and salivary gland sporozoites. Since X-crystallographic evidence implicates two amino acids of MyoA (E6 and phosphorylated S19) in MyoA kinetics, authors test the functional relevance of these two amino acids on motility by engineering point mutations in E6 and S9. E6 is substituted and the importance of S9's phosphorylation is interrogated by generating a phosphomimetic mutant (S19D) to mimic constitutive phosphorylation and an unphosphorylatable mutant (S19A). The mutants were taken through the entire lifecycle.

Data demonstrate that S6 mutations decrease the speed but not the fraction of motile sporozoites that is motile. This is interpreted to mean the S6 phosphorylation status is important for force generation. Force generation is assayed directly through an elegant bead-pulling assay.

The manuscript is generally well written and results are clearly described. There should be clearer explanation for the specific focus of E6 and S19.

Response: We thank the reviewer for the constructive comments below and the appreciation of our work.

**Major comments**

1) ookinete motility should be determined. Since the MyoA-based motor is required for ookinete movement, the effect of E6 and S19 mutations on ookinete motility should be established. Oocyst numbers are only an indirect measure and their being normal cannot necessarily be assumed to imply that ookinete motility is unaffected.

A: We now conducted three different motility assays from all mutants and found, to our positive surprise, that there is indeed a consistent difference in migration and speed. Especially surprising was the faster speed and higher percentage of motility of the S19D mutant. We included this data in Figure 3 and shifted part of Figure 3 into a new Figure 4. These experiments in our view now make for a very nice round story and we really thank the reviewer for making us do them. We adapted abstract and introduction accordingly and describe the results in lines 195--198. We also included an additional section in the discussion (lines 380-390) to put the results into perspective.

2) The delay in patency of S19A sporozoites is attributed, in part, to defective hepatocyte invasion in vivo. In vitro data suggest that sporozoites movement through the skin will be reduced. Authors should determine sporozoite invasion rate and infectivity in vitro to obtain a more direct measure of the S19A substitution's effect on invasion and consequently infection.

A: We also performed these experiments which showed no difference in in vitro infection of HepG2 cells by S19A compared to WT sporozoites. We show the data in a new Supplementary Figure 3 and refer to it in the text (lines 257-262), which reads: To examine liver cell infection we exposed HepG2 liver cells to WT or S19A sporozoites and investigated their capacity to form liver stages. This showed no difference in the number of infected liver cells or the size of developing liver stages between the two lines (**Supplementary Fig 3**). While this does not strictly exclude that S19A mutants have a defect in liver infection, which *in vivo* is more complex than *in vitro,* the data suggests a defect in skin migration." We also added (also upon request of reviewer 1 a line about the importance of salivary gland load which reads: "This suggests that indeed the most important contribution to diminished transmission is the lower number of parasites present in the salivary gland." (line 255-258)

3) Data charts do not contain any error bars. This should be rectified. Fig 4A should have p-value since the difference in % motile hemolymph sporozoites appear significant.

A: We only included p values that indicate "significant" differences for clarity instead of all possible p values. We did now include the p-values where the eye would catch a difference but there is none after statistical analysis and also included them in Figure 4A, which is now Figure 5A

4) Gene-targeting figures in Supplementary Material (Supp Fig. 1A and Fig. 2A and 2B) are difficult to follow as cloning strategies are totally unclear. Without significantly more detail, it is impossible to understand how mutant lines were generated. One example, in Supp Fig 2A how was the pbMyoA5'ama recipient line produced and how are mutations introduced into it? Does negative selection lead to excision of the marker? Is there a homologous recombination event, if so between which sequences? In Supp Fig 2C why is an amplicon absent in 'Sel' lanes of WT

reconstitution, E6R, S19A, S19D but present in Recipient line? Supp Fig 2B appears to show a deletion is the C-terminus of MyoA deltaN mutant.

A: This is now considerably more detailed. We have reworked the gene targeting figures and specifically the legends to the figures to show now recipient line, transfected construct and resulting parasite line in one figure. Homologous recombination events are indicated by grey areas.

We noticed that the gene-targeting figure describing the generation of the recipient line PbMyoA5'ama figure was missing from the initial manuscript, which likely led to the confusion. We apologize and have now added the scheme and the genotyping data to Supplementary Fig 2 as panels A and B. From this scheme, it should be now clear that the line PbMyoA5'ama has a selection cassette integrated into the genome (leading to the amplicon of the 'sel' PCR). This line acts as the recipient line for the subsequent second transfection (now depicted in panel C, D, and D of Supplementary Fig 2), in which the native myoA promoter along with point mutations is reconstituted. We selected for integration by negative selection using 5'FC, thus the resulting point mutation lines lack a selection marker (and thus the amplicon of the 'sel' PCR).

Methods description of gel migration assays and force measurements should be clearer.

A: We appreciate that these are somewhat unusual assays and but can't really see how to make this much clearer.

5) Amount of MyoA protein in all mutants and reconstituted WT need to be established. Observed phenotypes could be due, in part, to reduced protein. Related, is there evidence for altered MyoA phosphorylation in the S19 mutants? Alternative phosphorylations in vivo can occur and it would be interesting to know this.

A: we totally agree that it would be ideal to have this data. However, working on sporozoites now for over 15 years, we are usually just happy to get decent single western blots and to do reliable comparative protein analysis is nearly impossible, especially if you need to rely on midgut sporozoites (as salivary gland sporozoites of S19A are hard to obtain due to their low numbers). We add a few lines in the discussion now to clearly label this shortcoming of the study based on the limits of our "model system", see lines 458-461, which read: "In addition, we note that we did not determine the amount of myosin in our different mutants and cannot fully exclude that some of the observed differences are due to changes in protein levels. Similarly, the use of alternative phosphorylation sites was not examined and could potentially influence the expressed mutant proteins."

6) In fig 3C growth rate of S19D should be compared with the WT recon not RL.

A: Thanks, for noticing, this was corrected. There is no significant difference between WT recon and S19D or any other of the point mutant lines.

7) Increased hemolymph/middgut ration of S19A is quite interesting. Authors state "motility is required for egress from oocysts". These data appear to contradict this

statement. Is there an implication that MyoA phosphorylation inhibits exit of sporozoites from oocysts, or that a different Myosin is required for egress than invasion?

A: Very good question: we interpret this as an accumulation of sporozoites in the hemolymph as they cannot get into the salivary glands. We could not see evidence for myoA being involved in exit from oocysts, although this is very hard to quantitatively do, if the difference is not black and white. We discussed the labelling of the panel (now Figure 4A) prior to submission and have now changed it to “hemolymph sporozoites” to avoid confusion.

Reviewer #2 (Significance (Required)):

The manuscript represents an advance in our understanding of the in vivo role of MyoA phosphorylation. Authors made creative use of MyoA mutagenesis to address the in vivo functions of specific amino acids fingered in vitro as playing an important role in MyoA dynamics. Their focus on salivary gland invasion and hepatocyte invasion is significant since these invasive steps are critical for the parasite's life-cycle. Some conclusions need to be shored up through more direct experimentation in an otherwise robust manuscript.

A: We thank this reviewer for the great suggestion and hope he/she agrees that the manuscript has improved by our doing those experiments that are feasible.

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

This manuscript examines the importance of a key myosin phosphorylation site in the regulation of parasite motility, throughout the lifecycle of malaria parasites. Previous in vitro work demonstrated that phosphorylation of S19 increases Myosin stroke speed at the expense of force. Subsequent work in P. falciparum also demonstrated that mutations impairing the effect of S19 mutations reduced merozoite invasion efficiency. Here, generate a selection of mutant transgenic parasites including S19A and phosphomimetic S19D and demonstrate that interference with phosphorylation at this site affects asexual growth, sporozoite invasion in mosquito and mouse, but interesting with no apparent effect on ookinete traversal of midgut wall. Of these effects, the most dramatic was a reduction in ability of S19A mutants to invade salivary glands, thus highlight an important role for myosin phosphorylation/tuning during this stage. Interesting a detailed analysis of both sporozoite speed, force generation and motility characteristics, demonstrate that both S19A and S19D reduce motility speed and S19D reduces force. Not only does the work succeed in characterise the role of Myosin tuning throughout the lifecycle for the first time, but also represents the first detailed attempt to dissect the linkage between the in vitro Myosin tuning state with the actual parasite speed and force. As such the work reveals a far more complex role for Myosin tuning than previously hypothesised and marks major step forward in our understanding of how this works across the lifecycle of malaria parasites.

The experimental data are extremely robust throughout and the paper offers a sophisticated analysis of key motility parameters and force generation. The majority of my comments are minor, and no additional experimental work is required. My only negatives with this manuscript is that there are key areas where the novelty of the

work here is undersold - most notably that this is first time parasite motility and force has been examined in relation to tuning state. This seems to be based on the assumption that fast myosin will definitely make fast parasites rather than a more nuance use of this in the control of parasites. This is not the case and the data presented here generally points to the importance of dynamic Myosin tuning rather than one or other state being preferrable for a given invasive stage. That is fascinating and quite distinct to previously proposed roles for tuning. Finally the only other major issue I have is with the statements regarding the relative importance of salivary gland invasion in evolution of the parasite. I explain more in comments below, but I really feel this should be removed as it is both speculative and distracting.

A: thanks for the generous comments and also for the good suggestions. As the experiments with ookinetes that are now added in response to the request of reviewer 2 show, it's indeed even more complicated. We tried to expand the text with an aim to clarifying as much as we can but without speculating too much. We toned down the evolutionary aspects, also in response to reviewer 1.

**Major**

• The experimental approaches are all robust, have adequate replication and statistical analysis and includes both classic phenotypic analysis of mutant parasites through transmission with some very elegant detailed analysis of both sporozoite force, speed and motility parameters.

• L251, L335 and abstract, Authors Identify the main barrier for sporozoites unable to phosphorylate S19 as being salivary gland entry and therefore this is the main obstacle against which malaria parasites must evolve their migration and invasion machinery. This sentence (and equivalent in significance statement) come across as a big and leap, particularly against the really solid work that leads to it. Whilst I think I follow the logic, I see this as quite unnecessary and speculative. I strongly feel the authors should change it. The invasion machinery evolved well before the malaria sporozoite (even if early hosts were insects) and there are likely numerous specialist adaptations and posttranslational modification vital for each transitional step. Instead the authors should consider focusing on how their data has demonstrate differential importance for S19 regulation across the lifecycle, and that regulation of MyoA force and speed clearly does not correlate directly with speed and force of parasite. This highlights the complexity of parasite motility and the need for complementary in vitro and in vivo (or in parasite) experiments.

A: We have now toned down the statement regarding salivary gland invasion and evolution dramatically, which we believe helps the manuscript, see also reviewer 1. The added paragraph on ookinetes also includes statements about the complex S19 phospho-tuning. (now lines 193ff and 380ff). We also completely changed the ending of the discussion which now reads "Regulation of motility across the life cycle requires complex modulations according to the environmental barriers faced by the different forms of the parasites. Entry into salivary glands emerges as a strong barrier in parasite transmission which the parasites need to overcome." (lines 466)

• L269 I think it is important to note that Blake et al demonstrates that an equivalent of the S19A mutation (K764E) causes an impairment of progression between

deformation and invasion. The models to explain this using force thresholds, based upon the findings in Robert-Paganin et al., whilst compelling are theoretical, and the links for parasite force generation and speed are actually only tested here for the first time. Its important that this previous gap in our knowledge is recognised and the important efforts to characterise the effect on parasite speed and force made here. The work here goes quite significantly beyond characterisation possible in merozoites, where levels of deformation were the main characteristic measured.

A: Thanks for this comment, we changed “revealed” to “suggested” in the statement refering to the article by Blake (line 358) and added a few statements to clarify this also in response to a request by reviewer 1 to go deeper into the E6R mutation. We stay somewhat on the careful side with our interpretation as we really don’t know how force/motility/invasion all correlates. The added sentences read: “Interestingly, the E6R mutation caused an increase in force production *in vitro* (Moussaoui et al 2020). While these results appear contradictory, they might not be, as an increase in force on a single molecule level does not necessarily have to scale to an observable increase in force on the cellular level, where multiple myosins and actin filaments come together to generate an ensemble force, which is measured in our assay (Brown+Bridgman 2003; Quadt et al 2016). The E6R mutation also caused a reduced speed of moving actin filaments, which might *in vivo* be correlated with a lower force.”, (lines 366-372). Also, the paragraph added about ookinetes was phrased to answer this comment (lines 380-390)

• L283 I think the discussion oversimplifies in several places, and in doing so skims over some of the most interesting findings. I think its important to recognise that the "fast myosin" is probably dynamically required for particular steps rather than S19P simply meaning faster sporozoites. Data also showed that the D mutant also results in slower sporozoite motility - so its actually the dynamic regulation that appears to be important for speed. In addition specific stages appear more sensitive to S19A than S19D suggesting being locked in S19P is less detrimental than locked in S19. Sentences like L283 here could be modified to better reflect this.

A: We totally agree and wanted to state this, but clearly didn’t do a good enough job. Indeed, it’s not the “fixed” but the “dynamic” state that is essential. We hope that this now comes over much better especially since the addition of the ookinete data (included in Figure 3) shows that the S19D moves FASTER. WE hence added a strong statement along these lines at the end of the results section: “Together with the data on ookinetes, we suggest that it is the turnover of phosphorylation on S19 that is important for optimal progression of the life cycle.” (lines 334-336).

**Minor**

Figure 2 clarify whether 2 independent cage experiments were carried out on separate days with initiated with independent passages.

A: done, reads now “Data derived from at least two independent cage feeds originating from independently infected mice” line 794

Fig 2e legend indicates statistical analysis were carried out on E, but there are no indicators as to result in figure.

A: p-values are now added.

L166 The comparison with wt/S19A is interesting as it appears there is a single outlier reading with high growth rate. It seems likely that there is a difference here but with insufficient power to distinguish it (in part by need to adjust for multiple comparison). It's a tricky one, but wording of the S19A growing as well as wt, could perhaps be changed to the more technical "was not significantly different to wt"? On a wider point these slow growers are interesting as they would likely be very susceptible to adaptations. Could the authors note how passage numbers were regulated for these lines (i.e. to avoid experiments with high passages, I am sure they already were for gam formation).

A: Indeed, we struggled with this interpretation too and are obviously limited with the numbers of repeats we can perform. As suggested, we now changed the text to "Interestingly, growth of E6R and S19A mutants was not significantly different from wild type, although S19A showed in three out of four infections a lower infection rate." (lines 190-192). As for passage numbers: We generated a stock of frozen aliquotes from clones and always used a fresh one for each infection, so passage number essentially is 1 for all. Prior to refreezing parasites are cycled through mosquitoes to make sure they don't lose any bits of genome associated with this part of live cycle. In that case passage number would be 2. Kind of different from *P. falciparum in vitro* culture.

L192 Can the author back calculate the likely difference in parasite number resulting in a 1 day delay? This will help contextualise the effect of delayed parasitaemia (e.g. XX days delay is equivalent to XX lower hepatic schizonts). Was any statistical analysis undertaken to look at this delay?

A: We now added this information to the sentence "…90% reduction of infectivity, i.e. one order of magnitude fewer infected hepatocysts"(line 247), now Figure 4C. The significance is shown by bold writing in Table 2, determine with the tests indicated in the legend to Figure 4, which is now indicated in the legend of the table.

261 "Although invasion of the red cells appears as the easiest of these tasks for the parasite 261 due to the short time and distance," I think this needs rewording. I don't think we can make assessments of "Easy" because it must happen quickly. Perhaps more appropriate to separate out discussion of migration and invasion. The former is clearly more straight forward for meros, but invasion likely is highly complex for both meros and sporozoites (and simpler for Ookinetes).

A: agreed. We changed "easy" to "straightforward" (now line 349). Please note that we immediately follow with an argument, why it is not at all "easy".

L292 Worth noting S19 was also identified as being differentially phosphorylated across compound 2 (PKG inhibitor) treated schizont lines https://www.nature.com/articles/ncomms8285 - again supporting role for cGMP/Ca2+ regulation.

A: thanks, now mentioned in discussion: "Furthermore, S19 was also identified as differentially phosphorylated in late blood stage parasites treated with the protein

kinase G inhibitor ‘compound 2’ (Alam et al., 2015) supporting a role for cGMP/calcium signaling in regulation of motility.” (lines 402-404).

L325 This was a very interesting experiment, worth expanding on why this swap is interesting and how it could be used. Highlight that MyoA forms complex comment on whether Pb lightchains likely to still bind.

A: Very good point, we added “However, this parasite line could be useful for future biochemical experiments investigating the interaction of e.g. myosin A with its light chains.” (now lines 452-454)

Reviewer #3 (Significance (Required)):

The key conceptual advance of this work is in extending our knowledge of the role of myosin tuning in motility and invasion of parasites, particularly those within the Apicomplexan phylum. Building on the finding that myosin phosphorylation can increases motor speed in vitro, and blocking this impairs P. falciparum merozoite invasion, the work examines this throughout the lifecycle. The most exciting findings are the relatively differential importance at different lifecycle stages- with a large impact during salivary gland invasion and no effect on midgut traversal. Finally the work to link tuning state to specific parasite motility characteristic is a novel step and suggests tuning plays a complex role that dose not just simply increase motility speed.

These findings will be of significant interest to those looking at the relationship between motor protein regulation and cellular phenotypes, host pathogen interactions and particularly those working on invasion and motility of apicomplexan parasites.

The work also provides some interesting technical innovation through its exploration of approaches to modify the MyoA locus. The demonstration that generic 3'UTR function poorly during transmission stages is an important finding and will be of significant interest to anyone undertaking genetic modification of parasite.

I have worked on motility and invasion in both rodent and human malaria parasites, predominantly on ookinetes and merozoites. This has included significant work on the studying regulation of the parasite motor complex in P. falciparum and more recently work on gliding and motility in P. knowlesi merozoites.

All the best,
Rob Moon

A: Thanks Rob, most appreciated.

**Referee Cross-commenting**

I agree with reviewer 2 point regarding ookinete motility. It would be interesting to assess this. Measurements would be quite a significant amount of additional work so as alternative, it would be important to at least note that if motility is impacted in ookinetes, it is not sufficient so as to reduce ookinete-> oocyst transition.

A: we agreed too and you were right, it was interesting to see that it's even more complicated. See our answer to reviewer 2 and the added data in Figure 3 and associated text.

My interpretation of the final constructs is that mutant MyoA being placed under endogenous MyoA promoter, not AMA1 promoter as described by Reviewer 1. I had thought same on first read, and reviewer 3 also raises comments about difficulty in understanding cloning screen. I think the authors need to improve how this complex strategy is explained.

A: We expanded this section, thanks for flagging this, making these first lines was a large amount of time and we hope it will help others to save time in future.

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. Referee #2 has some further suggestions to improve the manuscript, I ask you to address in a further revised manuscript.

The manuscript needs also 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' and put it after the Acknowledgements section.

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

13) Please provide a title of not more than 100 characters (including spaces).

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

15) Please include 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 EV1' and change the callouts accordingly. Please provide the legend as a text file and ZIP it together with the movie file, and upload these as one folder. Finally, please remove the legend for the supplementary movie from the manuscript file.

17) Please provide the Supplementary Tables in an Appendix file (naming these 'Appendix Table Sx'). Please provide the Appendix file with Table of contents and page numbers. Finally, please change the callouts of the tables in the main text file and remove the supplementary tables from the manuscript file.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- three to four short one sentence 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:

The authors have adequately dealt with my previous concerns and the additional experiments and updated interpretations strengthen the story of the manuscript.

---
Referee #2:

This revision results in an excellent manuscript that presents novel and interesting findings on the effect of MyoA phosphorylation on ookinete and sporozoite motility. Authors conducted additional experiments and re-worked figures to provide missing information, in response to previous comments. The work represents significant progress in the mechanistic understanding
of MyoA kinetics and their impact in vivo.
Minor comments:
1)inclusion of PbMyoA geneID
2)Discussion mentions that S19 phosphorylation status was found to be PfPKG dependent in Alam et al 2015. In this context, it could be noted that Govindasamy et al 2018 (PMID: 30323024) predicted MyoA to be a direct substrate of PfPKG, and that S19 lies in the context of PfPKG's consensus phosphorylation site reported in PMID: 30323024.

---
Referee #3:

The authors have taken on board comments raised through the review commons review process. This included two significant new additional experiments suggested by a reviewer. These make excellent additions to the paper and further strengthens the work considerably. I recommend the work for publication in EMBO reports.

Referee #1:

The authors have adequately dealt with my previous concerns and the additional experiments and updated interpretations strengthen the story of the manuscript.

THANKS !!

---

Referee #2:

This revision results in an excellent manuscript that presents novel and interesting findings on the effect of MyoA phosphorylation on ookinete and sporozoite motility. Authors conducted additional experiments and re-worked figures to provide missing information, in response to previous comments. The work represents significant progress in the mechanistic understanding
of MyoA kinetics and their impact in vivo.

THANKS !!

Minor comments:
1)inclusion of PbMyoA geneID

Done (also for PfMyoA) lines 139 and 140

2)Discussion mentions that S19 phosphorylation status was found to be PfPKG dependent in Alam et al 2015. In this context, it could be noted that Govindasamy et al 2018 (PMID: 30323024) predicted MyoA to be a direct substrate of PfPKG, and that S19 lies in the context of PfPKG's consensus phosphorylation site reported in PMID: 30323024.

Thanks for suggestion, we included a statement and the new reference along those lines in the suggested part of the discussion, lines 358 - 361

---

Referee #3:

The authors have taken on board comments raised through the review commons review process. This included two significant new additional experiments suggested by a reviewer. These make excellent additions to the paper and further strengthens the work considerably. I recommend the work for publication in EMBO reports.

THANKS !!

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 few editorial requests I ask you to address in a final revised manuscript:

- Please reduce the number of keywords on the title page to 5.

- 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 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. Presently, some digrams have no statistic (e.g. 2F, 3f, 4A, 5B or S4) or partial statistics (e.g. 4B or S3B).

- Please make sure that all the funding information is entered into the online submission system and is complete and similar to the one in the manuscript text file. Presently, German Center for Infection Research, DZIF (TTU 03.813), has been entered in the submission system, but is not in the acknowledgements.

- Please select an answer in the author checklist for the section on antibodies.

- 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.

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

The authors have addressed all minor editorial requests.

Prof. Friedrich Frischknecht
Heidelberg University
Center for Infectious Diseases
Im Neuenheimer Feld 344
Heidelberg, Baden-Wuerttemberg 69120
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.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

**********

THINGS TO DO NOW:

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2022-54857V3 and be addressed to emboreports@wiley.com.

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-2022-54857 | |

**USEFUL LINKS FOR COMPLETING THIS FORM**

The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

**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? | Yes | Data Availability Section |

| 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:<br>- Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number<br>- Non-commercial: RRID or citation | Not Applicable | |

| 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 Tables |

| 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. | Yes | Materials and Methods |

| 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 |

| Plants and microbes | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| **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 | Acknowledment section |

**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. | Yes | Materials and Methods and References |

| 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 | Materials and Methods |
| 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?<br><br>If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification. | Not Applicable | |
| 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 |

## Ethics

| 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 approval** (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 | |

## Reporting

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 | |
| Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement? | Not Applicable | |
| 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 | |