EMBO reports

Conformational change of Plasmodium TRAP is essential for sporozoite migration and transmission

Friedrich Braumann, Dennis Klug, Jessica Kehrer, Gaojie Song, Juan Feng, Timothy Springer, and Friedrich Frischknecht
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Corresponding author(s): Friedrich Frischknecht (freddy.frischknecht@med.uni-heidelberg.de) , Dennis Klug (dennis.klug@sciencebridge.net), Timothy Springer (springer@crystal.harvard.edu)

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Review COMMONS

Transaction Report:

This manuscript was transferred to EMBO REPORTS following peer review at Review Commons.

Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

Plasmodium sporozoites rely on gliding motility to invade the salivary glands of the mosquito vector where they reside until being inoculated into the vertebrate host. Once in the vertebrate host, sporozoites again have to glide to pass through liver cells and reach the final host cell before exo-erythrocytic replication occurs. In sporozoites, thrombospondin-related anonymous protein (TRAP) is the key adhesin, linking the molecular motor to the substrate. TRAP and TRAP-like proteins contain on the extracellular side an inserted (I) domain that is responsible for substrate binding. The I domain can be in an open or closed state which can be fixed by mutating specific amino acids. Baumann et al. then assessed the effect of these states on gliding motility and infection. Whilst no effect was seen for both mutants with closed or open states until the production of haemolymph sporozoites, the number of salivary gland sporozoites were highly reduced in both mutants. The closed mutant was not able to be transmitted from mosquitoes to mice whilst the open mutant is severely impacted in transmission. Gliding of both mutant is highly impaired. Some of these phenotypes could be reverse by adding a reducing agent.

Major comments:

This is an elegant study with exhaustive experiments to address the research questions. One of the things I wondered about is the effect of exchanging amino acids. As far as I understood, structural information of integrins from other organisms informed the authors about positions that should be mutated and about the impact (or loss of it) on the 3D structure of the domain. Would it not be useful to run a structure prediction programme such as Alphafold on all the mutants to at least confirm stability of the domain structure upon mutation in silico?

Some of the concentrations of reducing agents tested are very high. Can the authors be sure that the parasites are not dead?

Minor comments:

- Please make sure that punctuation is correct (e.g. missing commas) and that there are

no other typing errors.
- Line 50: This sentence may imply mechanical rupture of oocysts rather than active egress of the sporozoite. Please re-phrase.
- Line 82: Do you mean a complexed Mg2+ ion?
- The introduction stops rather abruptly. Maybe a sentence of the significance of this study could be added.
- Line 361: 20 million parasites each? Please re-phrase to make it clearer.
- Line 363: Field of view needs to defined: What is the magnification used to observe exflagellation?
- Materials and Methods: Please write out reagent names for the first time.

Figures:

- Figure 1A is very small. The label font of the whole figure 1 is often too small.
- Figure 1D: Please list the components in the figure legend, e.g. blue: substrate etc.
- Figure 3: The figure title should be changed as there is no complete failure of sporozoites with fixed I-domains to invade salivary glands.
- Figure 5: for clarity, it would be easier if Figure C would not be squeezed into the legends of A and B.

2. Significance:

Significance (Required)

This study addresses in detail the mechanism of Plasmodium TRAP I domain in gliding motility and transmission. It builds on work from many years and groups including their own and contributes to deepen our understanding of TRAP-family proteins, gliding motility in Plasmodium sporozoites and maybe even in other Plasmodium stages or other Apicomplexan parasites.

This work is of interest to researchers in the field of Plasmodium mosquito stages and transmission as well as scientist who work on apicomplexan gliding motility and transmission.

I have previously worked on Plasmodium mosquito stages, but currently work on Toxoplasma gondii.

3. 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

4. *Review Commons* values the work of reviewers and encourages them to get credit for their work. Select 'Yes' below to register your reviewing activity at Web of Science Reviewer Recognition Service (formerly Publons); note that the content of your review will not be visible on Web of Science.

Web of Science Reviewer Recognition

Yes

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

This work seeks to investigate the effects on sporozoite motility of hypothesized conformational changes in TRAP's integrin domain. Previous structural studies suggested that the Integrin-like domain of TRAP assumes 'open' and 'closed' conformations induced by ligand binding. Here the authors hypothesize that TRAP's ability to dynamically switch between these states is crucial for sporozoite motility. The hypothesis is interesting and the authors elected to test it by 'fixing' TRAP in constitutively 'open' and 'closed' conformations by introducing Cys residues that are presumed to form disulphide bonds resulting in these states.

Major Comments

The approach is creative. The data that are presented are of high quality with adequate reproducibility. However, as written the manuscript does not provide a rationale for the specific substitutions they chose, how these substitutions are expected to lead to 'open' and 'closed' states, and how they mimic the natural route of TRAP transitioning

between the 'open' and 'closed' conformations.

Furthermore, there is no biochemical, biophysical or modeling data demonstrating that the introduced mutations impact the folding/unfolding of TRAP's I domain in the manner hypothesized. Therefore, it is difficult to interpret subsequent phenotypic data from the two mutant lines. While mutant parasites display defects in gliding motility, these defects are unexpected, perhaps pointing to alternative explanations - such as aberrant inter- or intra-molecular disulphide bonding in TRAP's extracellular domain.

About 10% of mutant sporozoites assumed to be in a constitutively 'closed' conformation display gliding motility and they move faster that WT. Yet this mutant did not cause patency in mice when introduced either via mosquito bite or IV injection. In contrast, the mutant assumed to be in an 'open' conformation displayed no motility in vitro but was able to infect mice (albeit at significantly reduced compared to controls). Presumably these data suggest that the in vitro gliding motility assays used are insufficient for testing the effect of these mutations on motility that is relevant in vivo. The model is that TRAP's interaction with extracellular ligands stabilizes its 'open' position. This suggests that motility assays conducted with extracellular matrix eg Matrigel are a more appropriate test of motility.

Experiments with DTT are difficult to interpret since there is once again no biochemical evidence that this treatment leads to a change in conformation of TRAP. DTT's effect on motility of the mutant could be non-specific. Overall, conclusions that are presented need to be supported by more data.

2. Significance:

Significance (Required)

Sporozoite motility is a prerequisite for infection by Plasmodium of the liver. A better mechanistic understanding of this process is significant for our understanding of the first step of malaria infection. TRAP is the major adhesin on the sporozoite surface and its loss abrogates sporozoite motility. The authors are to be commended for undertaking a challenging study.

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

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

4. *Review Commons* values the work of reviewers and encourages them to get credit for their work. Select 'Yes' below to register your reviewing activity at Web of Science Reviewer Recognition Service (formerly Publons); note that the content of your review will not be visible on Web of Science.

Web of Science Reviewer Recognition

Yes

Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

This paper builds on previous structural studies from the Springer lab, which show that the I-domain of the malaria sporozoite surface protein TRAP can switch between two conformations ("open" and "closed"), similar to what happens with the I domain of integrins. In the current study the authors explore whether the open and closed conformations of TRAP are important for sporozoite migration and infectivity by generating parasites expressing each form locked in place using introduced disulfides. Locking TRAP into either form inhibits sporozoite gliding, invasion of the mosquito salivary glands and transmission from mosquito to mouse. The addition of low levels of a reducing agent can partially rescue these effects with the open form, which the authors suggest points to a requirement for TRAP to undergo a switch between its open and closed states to support these key processes in the parasite's life cycle.

The paper is well written and the data and methods are clearly presented.

Major Comments:

Lines 109-115: The entire paper relies on the premise that the introduced disulfides

lock the I domain of TRAP in either the open or closed conformation. In the absence of experimental proof that this is the case, it would be helpful to the reader to have more detail on how this can be confidently inferred from the previous work on integrins -- perhaps as a supplementary figure.

The partial rescue of motility in the S210C/Q216C parasites by 50-100mM DTT is a VERY indirect approach to testing whether the conformational switch between the open and closed states is functionally important. The authors are appropriately reserved in their conclusions, but wildtype parasites are ultimately a better (less confounded) comparator; the authors may want to stress this more.

The implications of the current results for the authors' previous stick-and-slip model of sporozoite motility should be discussed.

Minor Comments:

The authors are experienced in the use reflection interference contrast microscopy to visualize attachment of the parasite to the substrate. Did they do any RICM on the mutants? Although not necessary for the paper, this would be a good way to support the interpretation of some of their results (eg, lines 185-190).

Line 127: Figure 2A does not show "no growth difference to wild type mice"

Line 154: "higher, but not significantly higher" - if the data don't meet the significance threshold being used, they cannot be called higher

Line 186: "showed nearly no floating parasites compared to the controls S210C and cFluo" - cFluo and S210C/Q216C show the same amount of floaters in Fig 5B. Also, S210C/Q216C HLS show similar levels of floaters to both controls in 2A.

Fig S2 defines unproductive gliding to include waving, but Figs. 5 and S3 scores waving as a separate category

Fig S3B and Fig 5D,E appear to be the same data presented twice

Line 304: "the inability of sporozoites with the TRAP I domain to migrate" (?)

Line 226: please explain what the + > ++++ qualitative descriptors signify in the tables and how they were scored

Lines 282, 312: the authors should mention here the extensive work that has been done on efficacy of viral vaccines directed against a particular conformational state of the immunogen

Fig 2B: The labels above the lanes are incomplete and therefore confusing; suggest sticking to the same nomenclature as in 2A

Typographical errors on lines: 58 (space missing), 80-81 (parentheses), 155 (Figure misspelled), 188 (expressing the closed (S210C/F224C)), 306 (comma after both)

Referees cross-commenting

Since all three reviewers questioned whether the introduced disulfides would have the assumed effects on I domain structure, the authors should provide a stronger rationale for this assumption or -- as suggested by reviewer 2 -- some actual data to support it. Reviewer 2's comment about the extracellular ligands available for the parasite to bind to in the gliding assay and whether this could influence the outcome (and relevance to what occurs in vivo) also deserves consideration.

2. Significance:

Significance (Required)

This is an elegant study that contributes important new information to our understanding of apicomplexan parasite motility and the function of the TRAP protein. The results will be of significant interest to those who study parasite motility, and likely also to those who study the role of integrins in cellular adhesion and signaling. The data nicely connect what was previously known about conformational changes in integrins with parasite adhesion to the substrate and motility.

I have expertise in the area of parasite motility.

3. 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

4. *Review Commons* values the work of reviewers and encourages them to get credit for their work. Select 'Yes' below to register your reviewing activity at Web of Science Reviewer Recognition Service (formerly Publons); note that the content of your review will not be visible on Web of Science.

Web of Science Reviewer Recognition

Yes

Full Revision

Manuscript number: RC-2022-01648
Corresponding author(s): Friedrich Frischknecht

[Please use this template only if the submitted manuscript should be considered by the affiliate journal as a full revision in response to the points raised by the reviewers.

If you wish to submit a preliminary revision with a revision plan, please use our "Revision Plan" template. ***It is important to use the appropriate template to clearly inform the editors of your intentions.]***

1. General Statements [optional]

Dear David,

Thanks for overseeing the review at Review Commons of our paper entitled "Conformational change of the Plasmodium TRAP I domain is essential for sporozoite migration and transmission of malaria". We have now prepared a revision and will forward this to partner journals. The goal of our study was to understand the role of the I domain of the Plasmodium adhesin TRAP in parasite motility and tissue invasion. Our paper follows a three decade long effort to understand this important protein of the malaria parasite. TRAP is expressed in Plasmodium sporozoites, the infective forms of the malaria parasite transmitted by mosquitoes. Along with CSP, from which the first and currently only licensed malaria vaccine was derived, TRAP is an essential surface protein and vaccine candidate. TRAP was cloned in 1988 (Robson et al., Nature), shown to bind heparan sulfates (Muller et al., EMBO J 1993) and liver cells (Robson et al., EMBO J 1995) and was one of the first genes deleted from the Plasmodium genome (Sultan et al., Cell 1997). This showed that TRAP is important for parasite motility, tissue and host cell invasion. Mutations in the cytoplasmic tail (Kappe et al., JCB 1999) and the extracellular I domain (Matuschewski et al., EMBO J 2002) showed that the former is important for motility and the latter for host cell invasion.

We have previously shown that deletion of the I domain leads to immotile and non-invasive parasites and that the I domain can be replaced by I domains from other species, which affected both migration and invasion (Klug et al., eLife 2020). Importantly, crystal structures predicted that the I domain could appear in an open and a closed conformation (Song et al., PNAS 2012) and suggested that a switch between the two could affect motility. In this paper we experimentally addressed this suggestion by introducing disulfide bonds to stabilize the I domain in the open or closed conformation. Strikingly, this revealed that parasites expressing the respective conformation fixed TRAP had difficulties to migrate and were not infectious. This provides further evidence, that the I domain of TRAP is important for both migration and invasion and strongly suggests that a dynamic interplay between the two conformations is important for both. Our work has thus also implications on understanding possible binding partners of TRAP and for vaccine design.

Full Revision

With the revision we have addressed all the points raised by the reviewers, who in general appreciated our study for its molecular mechanistic insights into TRAP function. In addition to changes in the text, we added structural data and AlphaFold predictions of the mutants. Please find our point by point rebuttal below.

2. Point-by-point description of the revisions

This section is mandatory. Please insert a point-by-point reply describing the revisions that were already carried out and included in the transferred manuscript.

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

Summary:
Plasmodium sporozoites rely on gliding motility to invade the salivary glands of the mosquito vector where they reside until being inoculated into the vertebrate host. Once in the vertebrate host, sporozoites again have to glide to pass through liver cells and reach the final host cell before exo-erythrocytic replication occurs. In sporozoites, thrombospondin-related anonymous protein (TRAP) is the key adhesin, linking the molecular motor to the substrate. TRAP and TRAP-like proteins contain on the extracellular side an inserted (I) domain that is responsible for substrate binding. The I domain can be in an open or closed state which can be fixed by mutating specific amino acids. Braumann et al. then assessed the effect of these states on gliding motility and infection. Whilst no effect was seen for both mutants with closed or open states until the production of haemolymph sporozoites, the number of salivary gland sporozoites were highly reduced in both mutants. The closed mutant was not able to be transmitted from mosquitoes to mice whilst the open mutant is severely impacted in transmission. Gliding of both mutant is highly impaired. Some of these phenotypes could be reverse by adding a reducing agent.

Major comments:
This is an elegant study with exhaustive experiments to address the research questions. One of the things I wondered about is the effect of exchanging amino acids. As far as I understood, structural information of integrins from other organisms informed the authors about positions that should be mutated and about the impact (or loss of it) on the 3D structure of the domain. Would it not be useful to run a structure prediction programme such as Alphafold on all the mutants to at least confirm stability of the domain structure upon mutation in silico?

A: Thanks for this suggestion, we added the crystal structure models of P. falciparum and P. vivax as well as the Alphafold models of the open and closed state of P. berghei TRAP as new panels in Figure 1 and Supplementary Figure 1, added some corresponding text to the results, materials and methods, and the figure legend.

Full Revision

Some of the concentrations of reducing agents tested are very high. Can the authors be sure that the parasites are not dead?

A: We used sporozoites expressing GFP in the cytosol and did not see loss of fluorescence. While this does not rule out that the parasites are not alive anymore, it shows that the plasma membrane was not compromised. Importantly, when using the more moderate concentrations that rescued motility, wild type parasites were also moving, showing that the parasites were alive.

Minor comments:
- Please make sure that punctuation is correct (e.g. missing commas) and that there are no other typing errors.

A: We carefully read and edited the manuscript again and hopefully corrected all those errors.

- Line 50: This sentence may imply mechanical rupture of oocysts rather than active egress of the sporozoite. Please re-phrase.

A: rephrased, it now reads “egress” instead of “burst”. (now line 52)

- Line 82: Do you mean a complexed Mg2+ ion?

A: Yes, this sentence was revised. (now line 86)

- The introduction stops rather abruptly. Maybe a sentence of the significance of this study could be added.

A: we added the following: “Our results suggest that active change between the open and closed conformation of the TRAP I domain is essential for sporozoite invasion of salivary glands and transmission from mosquitoes to mammals.” (line 109-112).

- Line 361: 20 million parasites each? Please re-phrase to make it clearer.

A: done, it now reads: “…into each of two naïve mice…”.(now line 399)

- Line 363: Field of view needs to defined: What is the magnification used to observe exflagellation?

A: done, it now reads: “as observed with a Zeiss Axiostar using a 100x objective lens – revealing 300-400 red blood cells per field of view) – now line 400-401.”

- Materials and Methods: Please write out reagent names for the first time.

A: done, e.g. “phosphate buffered saline” for PBS; line 418; Roswell Park Memorial Institute medium 1640 for RPMI; line 427; as well as for DTT and TCEP (lines 438, 439), BSA (line 443), etc

Figures:
- Figure 1A is very small. The label font of the whole figure 1 is often too small.

A: We intentionally kept Figure 1A small as most readers will be familiar with the life cycle but increased font size as much as possible throughout the figure including Figure 1B, which we needed to shrink in the new design to accommodate the Alphafold structures.

- Figure 1D: Please list the components in the figure legend, e.g. blue: substrate etc.

A: done, now lines 579-584

- Figure 3: The figure title should be changed as there is no complete failure of sporozoites with fixed I-domains to invade salivary glands.

A: done, it now reads: “…show strong reduction in salivary gland invasion”, line 627

- Figure 5: for clarity, it would be easier if Figure C would not be squeezed into the legends of A and B.

A: We agree this is a tricky arrangement, but when composing the figure, we tried many different ways, which were all unsatisfactory too, leading to either too awkward an arrangement or too much white between the panels. We hence, despite anticipating this critique, decided on this arrangement and would hence like to keep it. However, we changed the color of the box around C so that it is marked more differently.

Reviewer #1 (Significance (Required)):

This study addresses in detail the mechanism of Plasmodium TRAP I domain in gliding motility and transmission. It builds on work from many years and groups including their own and contributes to deepen our understanding of TRAP-family proteins, gliding motility in Plasmodium sporozoites and maybe even in other Plasmodium stages or other Apicomplexan parasites.

This work is of interest to researchers in the field of Plasmodium mosquito stages and transmission as well as scientist who work on apicomplexan gliding motility and transmission.

I have previously worked on Plasmodium mosquito stages, but currently work on Toxoplasma

gondii.

A: Thank you for your appreciation of our study, the mechanistic insights it gives into gliding and transmission, and the helpful critique.

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

This work seeks to investigate the effects on sporozoite motility of hypothesized conformational changes in TRAP's integrin domain. Previous structural studies suggested that the Integrin-like domain of TRAP assumes 'open' and 'closed' conformations induced by ligand binding. Here the authors hypothesize that TRAP's ability to dynamically switch between these states is crucial for sporozoite motility. The hypothesis is interesting and the authors elected to test it by 'fixing' TRAP in constitutively 'open' and 'closed' conformations by introducing Cys residues that are presumed to form disulphide bonds resulting in these states.

Major Comments
The approach is creative. The data that are presented are of high quality with adequate reproducibility. However, as written the manuscript does not provide a rationale for the specific substitutions they chose, how these substitutions are expected to lead to 'open' and 'closed' states, and how they mimic the natural route of TRAP transitioning between the 'open' and 'closed' conformations.

A: We now add a more detailed rationale at the very start of the results section, which now reads: “We used closed and open structures of TRAP from *P. falciparum* and *P. vivax*, respectively (Song et al., 2012), to design cysteine mutations in *P. berghei* (Fig. 1E-J). The TRAP I domain of *P. berghei* is 42 and 48% identical in sequence to those of *P. vivax* and *P. falciparum*, respectively; lower identity was used for successful introduction of a disulfide into integrin αI domains (Shimaoka et al., 2001; Shimaoka et al., 2003). Between the open and closed TRAP I domain conformations, the I domain α7-helix pistons 9 Å relative to the neighboring β6-strand; therefore, disulfides introduced between these two structural elements can stabilize one state over the other, as previously pioneered in integrin I domains. β6-strand and α7-helix residues with Cβ atoms within 5 Å of one another in structures of the two states were chosen for mutation to cysteine (Fig. 1G&H). *P. falciparum* TRAP-Fc fusions with homologous cysteine mutations were well expressed in mammalian cells (Koksal et al,, 2013). Using the TRAP sequence alignment (Fig. 1F), homologous residues in P. berghei TRAP were mutated to cysteine to stabilize the closed conformation (Ser-210 and Phe-224 in the S210C/F224C mutation) and the open conformation (Ser-210 and Gln-216 in the S210C/Q216C mutation). AlphaFold (Mirdita et al 2022) predicted that the *P.berghei* TRAP I domains were well folded and assumed the desired conformations with formation of the mutationally introduced disulfide bonds (Fig. 1I&J, Fig S1). The I domain of *P. berghei* is 42 and 48% identical in sequence to those of *P. vivax* and *P. falciparum* in which the open and closed conformations are defined, assuring highly confident modeling (Song et al., 2012). Cysteines were substituted

in positions that were sufficiently close in one conformation to form a disulfide but not in the alternative conformation..” lines 137-138 and also provided a detailed view of the structures in Figure 1.

Furthermore, there is no biochemical, biophysical or modeling data demonstrating that the introduced mutations impact the folding/unfolding of TRAP's I domain in the manner hypothesized. Therefore, it is difficult to interpret subsequent phenotypic data from the two mutant lines. While mutant parasites display defects in gliding motility, these defects are unexpected, perhaps pointing to alternative explanations - such as aberrant inter- or intra-molecular disulphide bonding in TRAP's extracellular domain.

A: The ability of such mutations to give biologically meaningful results has already been demonstrated in integrin I domains, where ligand-binding affinity was measured. We now describe the rationale in more detail, and demonstrate that the cysteines are recognized by Alphafold to yield well-folded domains that are stabilized in the desired conformations. These are presented as new panels in Figure 1 and the new Supplementary Figure 1.

About 10% of mutant sporozoites assumed to be in a constitutively 'closed' conformation display gliding motility and they move faster that WT. Yet this mutant did not cause patency in mice when introduced either via mosquito bite or IV injection. In contrast, the mutant assumed to be in an 'open' conformation displayed no motility in vitro but was able to infect mice (albeit at a significantly reduced rate compared to controls). Presumably, these data suggest that the in vitro gliding motility assays used are insufficient for testing the effect of these mutations on motility that is relevant in vivo. The model is that TRAP's interaction with extracellular ligands stabilizes its 'open' position. This suggests that motility assays conducted with extracellular matrix eg Matrigel are a more appropriate test of motility.

A: The reviewer raises interesting points. There are several types of sporozoite motility assays available, 2D on glass, 2D on ligands (e.g. Matrigel), 3D in polyacrylamide gels or Matrigel, and 3D in the skin. All of these have a number of advantages and disadvantages. 2D on glass is clearly the easiest, 3D in skin, clearly the most complex one. The advantage of the 2D on glass assay is that it reveals even tiny defects on motility that cannot be revealed in 3D assays as the enclosure of the parasite in a 3D matrix compensates for several defects, especially those in adhesion that are most relevant for our study here (see also e.g. Bane et al. Plos Path 2016, Ripp et al., EMBO Mol Med 2021). Hence, we believe that for understanding the role of adhesion in motility the 2D assay is highly valuable because it represents a minimal system. In contrast, the complex 3D environments, are closer to the natural situation but are less useful in our specific case. The fact that the mutants are severely limited in vivo (in the mosquito where they are blocked at the 2D surface of the salivary gland) suggests that the assays are highly relevant. We take this comment as a motivation to write a review/opinion like paper in the near future but feel it would distract if added here to e.g. the discussion.

Experiments with DTT are difficult to interpret since there is once again no biochemical

evidence that this treatment leads to a change in conformation of TRAP. DTT's effect on motility of the mutant could be non-specific. Overall, conclusions that are presented need to be supported by more data.

A: We are aware of this and hence already discussed these potential shortcomings of our study in the discussion. Importantly: there is no known specific ligand to the TRAP I domain. Hence, we believe that biochemical tests would not justify the effort it would take to express the different domains.

Reviewer #2 (Significance (Required)):

Sporozoite motility is a prerequisite for infection by Plasmodium of the liver. A better mechanistic understanding of this process is significant for our understanding of the first step of malaria infection. TRAP is the major adhesin on the sporozoite surface and its loss abrogates sporozoite motility. The authors are to be commended for undertaking a challenging study.

A: We thank the reviewer for appreciating our efforts, the advance provided, and for the constructive critique.

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

This paper builds on previous structural studies from the Springer lab, which show that the I-domain of the malaria sporozoite surface protein TRAP can switch between two conformations ("open" and "closed"), similar to what happens with the I domain of integrins. In the current study the authors explore whether the open and closed conformations of TRAP are important for sporozoite migration and infectivity by generating parasites expressing each form locked in place using introduced disulfides. Locking TRAP into either form inhibits sporozoite gliding, invasion of the mosquito salivary glands and transmission from mosquito to mouse. The addition of low levels of a reducing agent can partially rescue these effects with the open form, which the authors suggest points to a requirement for TRAP to undergo a switch between its open and closed states to support these key processes in the parasite's life cycle.

The paper is well written and the data and methods are clearly presented.

Major Comments:

Lines 109-115: The entire paper relies on the premise that the introduced disulfides lock the I domain of TRAP in either the open or closed conformation. In the absence of experimental proof that this is the case, it would be helpful to the reader to have more detail on how this can be confidently inferred from the previous work on integrins -- perhaps as a supplementary figure.

A: We now added a statement on how the positions for the cysteines were selected and also a figure on structures and Alphafold prediction as also suggested by reviewers 1 and 2 as panels

in Figure 1.

The partial rescue of motility in the S210C/Q216C parasites by 50-100mM DTT is a VERY indirect approach to testing whether the conformational switch between the open and closed states is functionally important. The authors are appropriately reserved in their conclusions, but wildtype parasites are ultimately a better (less confounded) comparator; the authors may want to stress this more.

A: Thanks for appreciating our efforts to navigate the limitations of our experimental system. We have changed our wording (e.g. replaced “fixed state” with “stabilized state” in the discussion) and added cautionary statements, which now read “While DTT clearly does not only act on TRAP, these data nevertheless suggest that…” (lines 254-255); “While not providing final proof, this phenotype indicates…” (lines 272); “…antibodies that stabilize… could contribute to stopping parasite migration” (line 309)

The implications of the current results for the authors' previous stick-and-slip model of sporozoite motility should be discussed.

A: We added a few sentences including two references on this in the discussion: “Sporozoites glide in a stick-slip fashion…” (lines 311-316)

Minor Comments:

The authors are experienced in the use reflection interference contrast microscopy to visualize attachment of the parasite to the substrate. Did they do any RICM on the mutants? Although not necessary for the paper, this would be a good way to support the interpretation of some of their results (eg, lines 185-190).

A: We indeed hoped that the mutants would allow us to exploit these techniques as this would give much insight into the mode of migration. However, all our previous work on RICM was done with salivary gland derived sporozoites, which are robustly gliding. In contrast, those derived from the hemolymph or oocysts do not glide very well. While we can quantitatively image them at low magnification (large field of view) settings, we have consistently failed to get sufficient data on most of our advanced assays that require single sporozoite imaging such as RICM, TIRFM, and laser tweezer experiments. This, unfortunately, is a real limitation of the system as most interesting mutants (see also e.g. recent paper by Yee et al., Plos Pathogens 2022) are not entering the salivary glands.

Line 127: Figure 2A does not show "no growth difference to wild type mice"

A: we split the sentence in two with Figure 2A now only referring to mosquito infections (now line 151)

Full Revision

Line 154: "higher, but not significantly higher" - if the data don't meet the significance threshold being used, they cannot be called higher

A: Yes, the thing here is that we always got more from the open mutant and hence could do experiments but indeed the stats showed it not to be significant. We changed the sentence to read “…was not significantly higher…” (line 178-180)

Line 186: "showed nearly no floating parasites compared to the controls S210C and cFluo" - cFluo and S210C/Q216C show the same amount of floaters in Fig 5B. Also, S210C/Q216C HLS show similar levels of floaters to both controls in 2A.

A: Thanks for spotting this mix-up, we changed it to: “Interestingly, hemolymph sporozoites expressing the closed I domain (S210CF224C) showed more floating parasites suggesting reduced adhesion. Intriguingly, the few moving sporozoites of this mutant were also significantly faster than the corresponding controls.” (lines 208-210). We also pick up on this observation in the discussion in the new paragraph on stick-slip motility, lines 311-320) which links back to the Munter et al paper cited in line 319. This is in line with observations in mammalian cells where reduced ligand density also leads to less adhesion and faster migration.

Fig S2 defines unproductive gliding to include waving, but Figs. 5 and S3 scores waving as a separate category

A: We define it as unproductive motility, which we now changed into “unproductive movement” in the hope to be less misleading, clearly “lazy gliding” is motility while waving is no motility but some type of movement.

Fig S3B and Fig 5D,E appear to be the same data presented twice

A: Correct, we now state this explicitly in the figure legend to Figure S3B (now Figure S4B lines 788-792) where they are reproduced together with the matching controls

Line 304: "the inability of sporozoites with the TRAP I domain to migrate" (?)

A: “conformationally stabilized” was missing and has now been included (line 337)

Line 226: please explain what the + > ++++ qualitative descriptors signify in the tables and how they were scored

A: Now added in the legends to the table, lines 712-718 and 720, respectively.

Lines 282, 312: the authors should mention here the extensive work that has been done on efficacy of viral vaccines directed against a particular conformational state of the immunogen

A: We were naturally tempted to do this but feel that this could hype the study more than is justified by our data.

Fig 2B: The labels above the lanes are incomplete and therefore confusing; suggest sticking to the same nomenclature as in 2A

A: Modified as suggested

Typographical errors on lines: 58 (space missing), 80-81 (parentheses), 155 (Figure misspelled), 188 (expressing the closed (S210C/F224C)), 306 (comma after both)

A: Thanks, these were corrected.

CROSS-CONSULTATION COMMENTS
Since all three reviewers questioned whether the introduced disulfides would have the assumed effects on I domain structure, the authors should provide a stronger rationale for this assumption or -- as suggested by reviewer 2 -- some actual data to support it. Reviewer 2's comment about the extracellular ligands available for the parasite to bind to in the gliding assay and whether this could influence the outcome (and relevance to what occurs in vivo) also deserves consideration.

A: We have now added Alphafold predictions of the mutants (Figure 1) and also described better how we selected the mutations based on extensive work by the Springer lab (see newly added lines 117-139). We appreciate the idea of using extracellular ligands, however, all our previous assays (e.g. Klug et al. eLife 2020 and Perschmann et al., Nano Letter 2011) suggest that the parasites move on a large variety of surfaces at the same rate.

Reviewer #3 (Significance (Required)):

This is an elegant study that contributes important new information to our understanding of apicomplexan parasite motility and the function of the TRAP protein. The results will be of significant interest to those who study parasite motility, and likely also to those who study the role of integrins in cellular adhesion and signaling. The data nicely connect what was previously known about conformational changes in integrins with parasite adhesion to the substrate and motility.

I have expertise in the area of parasite motility.

A: We thank the reviewer for the constructive comments and appreciation of our work

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 #3 has some suggestions to improve the manuscript, I ask you to address in a 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

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 now request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator (in cc) will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

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. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams.

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) We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions and do not add author contributions to the manuscript text file. See also guide to authors:
https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

13) Please add up to 5 keywords to the manuscript and order the manuscript sections like this, using these names:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials and Methods - Data availability section - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends - Tables

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

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) I would suggest uploading the "Key Resources Table" in the methods section as 'Reagents and Tools table'. I have attached templates for that in word or excel format. Please upload the filled in table to the manuscript tracking system as a 'Reagent Table' file and remove the table from the manuscript text file. The example below shows how the table will display in the published article and includes examples of the type of information that should be provided for the different categories of reagents and tools. Please list your reagents/tools using the categories provided in the template and do not add additional subheadings to the table. Reagents/tools that do not fit in any of the specific categories can be listed under "Other":
https://www.embopress.org/pb%2Dassets/embo-site/msb_177951_sample_FINAL.pdf

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 addressed all of my previous concerns. Congratulations on a nice piece of work.

Referee #2:

The authors have addressed all my previous comments adequately and I believe they did also a good job in addressing the issues from the other reviewers. I am happy to see that they have added Alphafold predictions of the mutants and crystal structure models (that is useful for the first comment from Reviewer 2) to figure 1.

I am looking forward to seeing this nice piece of work published.

Referee #3:

The revisions have addressed my concerns. Authors should include the confidence level of the Alphafold predictions. There are some typos that should be corrected eg line 141.

***
Rev_Com_number: RC-2022-01648
New_manu_number: EMBOR-2023-57064V1
Corr_author: Frischknecht
Title: Conformational change of the Plasmodium TRAP I domain is essential for sporozoite migration and transmission of malaria

The authors have addressed all minor editorial requests.

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:

- There are three corresponding authors listed on the title page of the manuscript but only one is indicated as such in the submission system. Please clarify.

- Please provide the abstract written in present tense throughout.

- We plan to publish your manuscript in the Report format (as also indicated by you in the submission system), as there are not more than 5 main figures. For a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do this for your manuscript. For more details, please refer to our guide to authors: http://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

- 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. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams.

- 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, there is a discrepancy between project numbers for the NIH Grant HL 131729 (in manuscript) and HL 131728 in the submission system. Moreover, please provide one Acknowledgments section with all the funding information and remove the subheadings "Funding" and "Funding statement".

- Please make sure that all figure panels and Tables are called out separately and sequentially (main and EV items). Presently, a callout for Fig. 5F seems missing, all EV figure panels need separate callouts and Table 4 is called out before Table 1. Please check.

- Please upload the schematic summary figure as separate in jpeg or tiff format (with the exact width of 550 pixels and a height of not more than 400 pixels).

- 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.

Achim Breiling
Senior Editor
EMBO Reports

_____

***

Rev_Com_number: RC-2022-01648
New_manu_number: EMBOR-2023-57064V2
Corr_author: Frischknecht
Title: Conformational change of Plasmodium TRAP is essential for sporozoite migration and transmission

Heidelberg, May 15th, 2023

Dear Achim,

thanks again for overseeing the publication process of our paper and apologies for the delay in answering. Please find the revised version and a new Figure EV4 (with n indicated) as well as a new graphical abstract attached and answers to your queries below.
Looking forward to hearing from you
best wishes
Freddy

On Tue, May 2, 2023 at 3:56 PM Frischknecht, Freddy <Freddy.Frischknecht@med.uni-heidelberg.de> wrote:

Von: contact@emboreports.org
Gesendet: Dienstag, 2. Mai 2023 15:56:49 (UTC+01:00) Brüssel (Bruxelles, Brussels), Kopenhagen (København), Madrid, Paris
An: Frischknecht, Freddy
Betreff: Decision on Manuscript EMBOR-2023-57064V2| [RC-2022-01648] [REV]

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:

- There are three corresponding authors listed on the title page of the manuscript but only one is indicated as such in the submission system. Please clarify.

The three corresponding authors is correct as in our previous paper Klug et al., eLife 2020, doi: 10.7554/eLife.57572 and correctly reflects the collaborative nature of this project. I probably didn't understand that you can click multiple corresponding authors in the system, apologies.

- Please provide the abstract written in present tense throughout.

Now adapted to present tense.

- We plan to publish your manuscript in the Report format (as also indicated by you in the submission system), as there are not more than 5 main figures. For a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do this for your manuscript. For more details, please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

Now rewritten within one section "Results & Discussion" and different subheadings.

- 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. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams.

We added the n for Figure EV4, where they were missing on top of panel B (all numbers also apply to the other panels). However, we kindly ask to keep the bar diagrams even if the experiments are from less than 5 independent experiments (infected mosquito cages). Each independent experiment is made from multiple recordings of fields of views and record hundreds of cells. We played around with different ways (including dot plots) of visualizing the data and settled on this version as this gets the experimental outcome best across. While potentially doable for the top panels in Figure 5 A and B and for Figure EV4 panel B it generates impossible to grasp graphs for the other panels with the multiple (up to 5) different categories of movement patterns. It is also for optimizing visualization that we use SEM instead of SD, which I usually prefer to use for 'small' datasets.

- 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, there is a discrepancy between project numbers for the NIH Grant HL 131729 (in manuscript) and HL 131728 in the submission system. Moreover, please provide one Acknowledgments section with all the funding information and remove the subheadings "Funding" and "Funding statement".

The HL 13729 is correct.

- Please make sure that all figure panels and Tables are called out separately and sequentially (main and EV items). Presently, a callout for Fig. 5F seems missing, all EV figure panels need separate callouts and Table 4 is called out before Table 1. Please check.

This was implemented with table 1 now being called out earlier (line 148) and Figure 5F called out (line 237)

- Please upload the schematic summary figure as separate in jpeg or tiff format (with the exact width of 550 pixels and a height of not more than 400 pixels).

Please also see attached

- 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.

Thanks, we carefully went through the manuscript and added/adapted all the requested changes in track changes.

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.

Thanks so much again, please also do advise on our cover suggestion (we couldn't upload the high res version during submission).

best wishes
Freddy

Achim Breiling
Senior Editor
EMBO Reports

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.

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Yours sincerely,

Achim Breiling
Editor
EMBO Reports

****

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All further communications concerning your paper should quote reference number EMBOR-2023-57064V3 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-2023-57064V1

USEFUL LINKS FOR COMPLETING THIS FORM

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EMBO Reports - Author Guidelines
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Reporting Checklist for Life Science Articles (updated January

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
- 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

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:

  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

Materials

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

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

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	R+T Table, Table EV1

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.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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

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	Material and Methods, R+T Table

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?	Not Applicable	

Design

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

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

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figures, 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?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Material and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Material and Methods (Ethics statement)
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	Figures, 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 approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods (ethics statement)
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?	Yes	Figure legend 1
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Reference list