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The cortical microtubules of *Toxoplasma gondii* underlie the helicity of parasite movement

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MS TITLE: The cortical microtubules of *Toxoplasma* contribute to the helicity, but not the speed or persistence of parasite movement

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, all three reviewers are positive but they have collectively raised a number of criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Cortical microtubules are central cytoskeletal structures important for apicomplexan parasite morphology. In contrast to their eukaryotic counterpart cortical microtubules in apicomplexan parasites are ultra-stable against cold and detergent treatment. In this paper, the authors quantitatively investigated the morphology of cortical microtubules in a previously generated Toxoplasma gondii mutant (TKO) that lacks three microtubule-associated proteins. Depletion of all three proteins resulted in curtailed and often sparse cortical microtubules which was most dominant in maternal cells during daughter cell formation. While microtubules became more sensitive to cold treatment in mutant parasites compared to wildtype, the effect was reversible and mutant parasites could recover upon warmer temperatures. The authors further characterized the motility behaviour in TKO parasites in great detail and compared it to a conoid mutant. While depletion of these microtubule-associated binding proteins did not have an effect on the overall speed or run length, mutant parasites moved predominantly in linear paths compared to the characteristic helical motility observed in wild-type parasites. While TKO parasites could invade and egress host cells normally, they showed a reduced infectivity. Overall, the study highlights how changes in microtubule morphology affect both resistance to stress as well as parasite motility.

Comments for the author

I suggest the following modifications 1. Adding some additional mutants (eg. as suggested in point 10 by complementing the TKO with either of the three proteins). This could provide a more thorough study and hence a better match to the scope of JCS.
2. To allow full understanding of all potential readers, all figures should be in a colour-blind friendly way. Hence please change 3D-SIM images to a colour-blind friendly version.
3. While certain parasite strains might be well-known in the Toxoplama community, it might not be for the general cell bio/parasitology community.
Hence please provide short explanations to the used parasite lines and introduce them as soon as they are used in the paper (e.g. the strain RHΔhx was only explained in line 244, even though it was already part of Figure 1; RHΔh80 and Δdcx are only introduced in lines 220-224 while they are already part of Figure 2 and Figure 3A).
4. Methodology is not comprehensively described. How was the “sparse” phenotype described, and what microtubule length defined the “short” phenotype.
Also, methodology should be ordered in the same order as the experiments/results appear in the main text. Experimental setup for live invasion assay is described early on in the methods section while the corresponding results only appear in the final figure 4.
5. While the overall storyline is well-understood, there are some grammar or spelling mistakes that should be corrected (e.g Line 31: The cortical microtubules, which are ultra-stable, sprially arranged, and …). Also avoid too long sentences to provide better readability (e.g. sentence line 65 – 69).
6. The overall structure of the introduction is well-understandable and has a red thread. However, parts of the introduction are quite similar to the abstract (line 45 – 52 versus line 110 – 119). Readability would improve if parts of the introduction were rephrased.
7. Please provide exact numbers of the quantified the entry attempts (line 261 – 262) and plot them as a subpanel of Figure 4.
8. After cold-induced polymerization, a shift to 37°C allows microtubules to recover. However, according to Figure 1, panel E, this effect is not completely reversible as at R120 only around 70% of TKO parasites have normal microtubules. Hence the sentence in line 43 should be tuned-down a bit as the effect can only be partially recovered.
9. The authors showed nicely in Figure 4 that the TKO mutant has no defect in neither invasion nor egress. While they included the Δdcx parasite line for the invasion study that showed a prolonged invasion time, they should also include it for the egress study.
10. The authors characterized the motility patterns of the TKO mutant extensively in this paper. To get more insights into protein redundancy and protein hierarchies, complementing the TKO mutant with either of the three microtubule-associated proteins to see which protein can rescue the phenotype to restore microtubule length and motility would be a powerful addition to the study. Some complemented parasites seem already available.

11. As shown in their preceding publication, TLAP2 and TLAP3 bind to different regions of the cortical microtubules. While TLAP2 was shown to mostly localize across the full microtubule length except for a ring region at the apical end, TLP3 appeared to be only present at the apical end of cortical microtubules. How do the authors think TLAP2 and TLAP3 contribute to microtubule stability, also in combination with SPM1?
12. While the TKO has a very interesting phenotype considering motility patterns, the authors showed that depletion of the proteins does neither affect invasion nor egress of host cells. Hence, the authors might want to consider to change the order of their figures and put the current figure 3 as the final figure to hence end their publication with an intriguing finding in motility patterns upon microtubule disruption.
13. As shown in their preceding paper, TLAP3 expression is established early on during daughter cell formation. In contrast, the authors show in the current paper that microtubules are only disorganized in the mother but not the daughter cells during replication. Do the authors have an idea why microtubule organization is only affected in mother cell but not the daughter cells even though all cells share the same cytoplasm.

Figures:
- Figure 1, Panel C: Indicate clearly in the figure legend that the quantification in microtubule morphology refers to the mother cell but not the daughter cells.
- Figure 1: Have labels of subpanels aligned to the same side (TKO, Tub IMC1,…)
- Figure 3, panel A: increase the distance between the lines to separate the parameters also visually.
- Figure 3, panel B: How does the motility paths look like for RHΔhx cold-treated? Provide a negative control here.
- Figure 4: Statistical tests are missing.
- Figure 4, Panel B, D: The official scientific abbreviation for seconds is s not sec. Please change accordingly.
- Figure 4, Panel D: Plot the median as part of the graph instead of having it written below the graph.
- Figure 3, Figure 4: Exchange asterisks with exact p values.

Reviewer 2

Advance summary and potential significance to field

In this study, Tengganu/Arias Padilla/Munera Lopez and colleagues further characterize a Toxoplasma gondii mutant published by their team several years ago (Liu et al. 2016). This mutant (called TKO for triple knock-out) lacks 3 subpellicular microtubule associated proteins, namely SPM1, TLAP2 and TLAP3, causing major destabilization of the subpellicular microtubules (SPMTs). The SPMTs are an essential component of the parasite cytoskeleton involved in parasite morphology. While the TKO displays no defect in daughter cell formation, trajectory of movement is impaired in 3D Matrigel. TKO parasites partially lose the helicity of the movement usually displayed by parasites gliding in a 3D environment.

The authors report some interesting findings on a previously described mutant, TKO, in a beautiful paper (Lui et al, Mol Biol Cell 2016), which is here phenotypically refined. The microscopy images on fixed samples and live imaging video, are of high caliber and some are supported by solid quantifications. This focused analysis reveals that this mutant glides at normal speed but is altered in the helical movement, which is not significantly affecting invasion and egress. Some additional experiments would be important to validate the claims and the analysis could be extended to other mutants to consolidate and broaden the findings. The manuscript would also benefit from editing to improve readability.

Comments for the author

MAJOR COMMENTS:
1. The data obtained from the 3D Matrigel are solid: TKO parasites partially lose helicity of the movement, following a "straighter" path during motility. The authors link this loss of helicity to the

loss of the SPMTs but minimize the importance of alteration of parasite shape in this process. To firmly address if the absence of SPTMs is impacting or not on "banana-shape to the parasites, the authors should determine experimentally and quantitatively the parasite shape. This could be achieved by providing comparative morphological data on the TKO mutant and WT. Length, width and length:width ratio should be quantified as performed before (Leung et al. 2017) and ideally the curvature of the parasites should also be quantified. This would provide more robust data on the "banana-shape" of the parasites and assess if the TKO are "straighter" than WT parasites.

2. On the same 3D Matrigel experiment (Figure 3), the authors present an analysis of the motility for the TKO after cold treatment. A careful quantification of net displacement, run length, run speed, turning angle, dispersal ratio and pitch are required such that a proper comparison between TKO untreated and wild-type parasite can be achieved.

3. The analysis of SPMTs morphology after cold treatment has been performed by IFA on intracellular parasites while the 3D motility experiments are performed with extracellular parasites. It would be relevant assess the morphology of their SPMTs after cold treatment (in WT and TKO) and quantify the different phenotypes (WT-like / short-sparse / absent) by IFA on extracellular parasites.

4. TKO parasites present dramatically shorten/absent SPMTs. This structure is not only important for parasite shape, but it has also been hypothesized that micronemes are positioned and trafficked along them (Leung et al. 2017). Furthermore, it is stated in the text that there is “lower incidence of entry attempts (…) for the TKO”. It appears important to fully characterize the TKO mutant by performing quantitative microneme secretion-, host cell attachment- and invasion-assays.

5. Fig. 1C convincingly shows that daughter cell formation is not impaired in the TKO mutant. As stated in the manuscript, this could be due to other proteins stabilizing the nascent SPMTs. However, the role of SPM1, TLAP2 and TLAP3 (knocked out in the TKO) in daughter cell is not investigated. It is not clearly stated or shown if those three proteins are decorating the daughter cells SPMTs. The authors should provide this information by citing the literature or including the data on localization of the three proteins in daughter cells.

6. There are other relevant mutants published that display altered shape/SPMTs morphologies (Ma et al. 2007 10.1091/mbc.e07-04-0379 or Liu et al. 2016). These parasites either harbor either a mutation in the alpha-tubulin gene or overexpress TLAP2 and in both cases exhibit longer/straighter SPMTs that lead to longer and straighter parasites. A comparison of the trajectories of these mutants with TKO would be very complementary. Inclusion of these mutant parasites in the 3D Matrigel analysis reported here would reinforce the claims and give more weight to the work.

MINOR COMMENTS:
Some sentences are convoluted and would benefit to be shortened.
Figure 2 could be a supplementary figure. The pipeline used in this study for trajectory tracking is excellent but similar protocol have been published before (Leung et al. 2014).
The abstract is too long. Journal of Cell Science guidelines indicate that the abstract should be no more than 180 words.
Include statistics for graph in Fig. 4B and 4D.
Fig. 4B represents "time taken to disperse" but this is not what is described in the results section. In the text it is mentioned egress, not time to disperse.
Line 1: Toxoplasma gondii in full.
Line 31: Correct "spirally"
Line 35: please rephrase the sentence “In wild-type parasites, the cortical microtubules in mature parasites (…)” so it is not repetitive or simply delete "in wild-type parasites".
Line 53-55: the sentence is difficult to understand. What is the difference between "host cell entry" and "infection" ?
Line 65-69: the sentence is too long.
Line 66-67: "successful infection requires the parasite to travel over a long distance through tissues". If this is the case and it has been shown previously, a citation is needed.
Line 72-77: the sentence is too long. To be split to separate the citations and refer to them more precisely with explanatory sentences.
Line 78: “energy is injected into the system” to be rephrased and explained.

Line 80: “polymerization is likely to occur dynamically”, please include citation as extensive work has been done on intrinsic instability of apicomplexan F-actin.
Line 93: “helical, or more accurately,” is dispensable. Consider referring to “spiral pattern”.

Reviewer 3

Advance summary and potential significance to field

This manuscript from Tengganu, Hu and colleagues investigates the role of the cortical microtubules during motility and invasion of Toxoplasma gondii. The authors first characterize the organization of the microtubules in a triple knockout parasite line lacking three MT binding proteins and demonstrate a significance change in MT length and number. Using this line, the authors then investigate if the helical arrangement of the microtubules contributes to the helical trajectories of motile parasites. The authors elegantly demonstrate that the loss of MTs results in a change from helical to linear movement. The surprising and novel result is that many other parameters of motion, such as velocity and run-length, were not affected in the TKO parasites, which is unexpected and counter to what many in the field might have predicted as helical motion was seen as a crucial aspect of parasite movement. Thus, this paper expands to our understanding of MT function and parameters that govern motility in the parasite. In addition, the authors modified the previously developed 3D motility assay so that imaging and analysis are performed with DIC microscopy rather than fluorescence. Overall, this paper is well written and lacks jargon and it will be accessible to and of interest to readers both inside and outside the field of parasitology.

Comments for the author

Minor comments:
1) Motility in cold-treated parasites: On line 167 authors state that the cortical MT arrays are significantly restored after 20 minutes of temperature shift from 4C to 37C. The authors then go on to image motility in cold treated parasites at 37C (paragraph beginning on line 210). The authors state the imaging is done “immediately” after shift to 37C but it is unclear how long the imaging takes and if the MTs remain disrupted for the entire imaging period.
2) The authors have previously demonstrated that micronemes are associated with the cortical MTs, Leung et al 2017, PMID: 28331073. One would anticipate that the disruption to the microtubules would also lead to a chance in the localization of micronemes, however microneme secretion does not appear to be affected as parasite invasion was not disrupted. Did the authors evaluate the localization of micronemes to determine if (a) MTs are needed for apical localization of the micronemes and (b) if apical localization of micronemes is needed for secretion.

First revision

Author response to reviewers' comments

In the following Response to the reviews, the original words of the reviewers are in *italics*, and our responses in plain text. Text from our revised manuscript is included in "".

Reviewer 1

1. *Adding some additional mutants (eg. as suggested in point 10 by complementing the TKO with either of the three proteins). This could provide a more thorough study and hence a better match to the scope of JCS.*

We have added the 3D motility analysis of a single (*Δtlap2*) and a double microtubule mutant (*Δtlap2Δspm1*) and compared them with those for the wild-type and the TKO parasite. Please see below in response to point 10 for the rationale of choosing these two mutants.

2. *To allow full understanding of all potential readers, all figures should be in a colour-blind friendly way. Hence please change 3D-SIM images to a colour-blind friendly version.*

All fluorescence images are now displayed with a colorblind-friendly scheme.

3.*While certain parasite strains might be well-known in the Toxoplama community, it might not be for the general cell bio/parasitology community. Hence please provide short explanations to the used parasite lines and introduce them as soon as they are used in the paper (e.g. the strain RHΔhx was only explained in line 244, even though it was already part of Figure 1; RHΔh80 and Δdcx are only introduced in lines 220-224 while they are already part of Figure 2 and Figure 3A).*

These parasite strains are now introduced earlier in the text as suggested. RH*Δhx* is now introduced before Figure 1 (Line 115).

RH*Δku80* and *Δdcx* are introduced in Line 218-220 with Figure 3 and 4 (original Figure 2 and 3)

4.Methodology is not comprehensively described. How was the "sparse" phenotype described, and what microtubule length defined the "short" phenotype.

We have now described the definition of the two phenotypes in the main text. Line 122-126:
"The "short" and "sparse" phenotypes are defined as follows: "short"- microtubules at approximately normal density extend to less than 1/3 of the parasite body length and very few microtubules are longer than ~ 1/2 of the parasite body length. "sparse"- more microtubules are longer than 1/2 of the parasite body length but there are obvious gaps in the array."

Also, methodology should be ordered in the same order as the experiments/results appear in the main text. Experimental setup for live invasion assay is described early on in the methods section while the corresponding results only appear in the final figure 4.

Experimental setup for the egress, live invasion, and plaque assays have been moved to the later part of the methods section to be consistent with the order of the description in the main text.

5.While the overall storyline is well-understood, there are some grammar or spelling mistakes that should be corrected (e.g Line 31: The cortical microtubules, which are ultra-stable, sprially arranged, and …). Also avoid too long sentences to provide better readability (e.g. sentence line 65 - 69).

The spelling mistake has been corrected.

The long sentence in original line 65-69 has now been separated into two as follows:
Line 49- 53: "As obligate intracellular organisms, a successful infection requires the parasite to travel over a long distance through tissues with varying biochemical and biophysical properties [3] as well as to enter into host cells. The latter requires a shorter travel distance, but perhaps more finesse, as the host cell plasma membrane needs to remain intact to allow the parasite to use host cell resources for proliferation. "

6.*The overall structure of the introduction is well-understandable and has a red thread. However, parts of the introduction are quite similar to the abstract (line 45 - 52 versus line 110 - 119). Readability would improve if parts of the introduction were rephrased.*

The abstract has been extensively revised and shortened to be within the 180-word limit requested by *JCS*. The wording in the abstract is now distinct from that in the introduction.

7.Please provide exact numbers of the quantified the entry attempts (line 261 - 262) and plot them as a subpanel of Figure 4.

This information was already included in the original manuscript. The exact numbers of the

quantified entry attempts and the number of videos taken were stated in the paragraph preceding the original line 261-262. The information was also included as part of Figure 4D (current Figure 6G, N= observed entry attempts)

Line 324-331: “The number of entry events was recorded for four parasite lines: wild-type RHΔhx parental, TKO, wild-type RHΔku80 parental, and Δdcx parasite. We observed 58 events for RHΔhx in 10 videos, 40 for TKO in 13 videos, 56 for RHΔku80 in 9 videos, and 35 for Δdcx in 22 videos. The median speed of host cell entry was 25.5 seconds for RHΔhx, 22.5 seconds for the TKO, 25 seconds for the RHΔhxΔku80 and 33 seconds for the Δdcx parasites (Fig 6G). We also did not observe any notable differences among these parasite lines in the morphological changes (e.g. the formation and resolution of constrictions) that accompany host cell entry. ”

8. *After cold-induced polymerization, a shift to 37°C allows microtubules to recover. However, according to Figure 1, panel E, this effect is not completely reversible as at R120 only around 70% of TKO parasites have normal microtubules. Hence the sentence in line 43 should be tuned-down a bit as the effect can only be partially recovered.*

This sentence has been now removed from the abstract due to the extensive revision. The related sentence in introduction (line 95-97) has now been changed to: "Depolymerization in mature TKO parasites becomes even more pronounced upon the initiation of daughter construction or cold treatment, the latter effect being partly reversible."

9.*The authors showed nicely in Figure 4 that the TKO mutant has no defect in neither invasion nor egress. While they included the Δdcx parasite line for the invasion study that showed a prolonged invasion time, they should also include it for the egress study.*

We have now included calcium-ionophore-induced egress study for the *Δdcx* parasite line, the dispersal time of which is similar to its wild-type parental. The data is included as part of the dot plot in Figure 6E. Please note that we found that one data point for the egress assay for the RHΔhx parasite (wild-type parent of the TKO) was incorrectly included in the original dot plot. This has now been corrected.

10.*The authors characterized the motility patterns of the TKO mutant extensively in this paper. To get more insights into protein redundancy and protein hierarchies, complementing the TKO mutant with either of the three microtubule-associated proteins to see which protein can rescue the phenotype to restore microtubule length and motility would be a powerful addition to the study. Some complemented parasites seem already available.*

In the complemented parasite that the reviewer referred to, the expression of the mEmerald-TLAP3 in the TKO (*Δtlap2Δspm1Δtlap3*) was ectopic and in a mixed population. While it was useful in showing that the expression of the apically localized TLAP3 restored the stability of the microtubule at the parasite apical region, it is not optimal for the motility study, which is a population-level assay. We therefore decided to use the *Δtlap2Δspm1* and *Δtlap2* mutants to address this point.

We have now included the analysis of microtubule morphology (Figure 2) and 3D motility (Figure 5) for the *Δtlap2, Δtlap2Δspm1,* and *Δtlap2Δspm1Δtlap3* mutants along with the wild- type parent. We found that the impact on trajectory helicity is very much in line with the extent of perturbation of the microtubule array. The loss of TLAP2 alone results in mild disruption of the microtubule array and very modest change in helicity of the movement. The loss of TLAP2 and SPM1 together results in much more pronounced defects in microtubule array as well as significantly more linear movement.

Please note that in this second set of 3D motility experiments, a new batch of Matrigel was used. It is well known that the mechanical properties of Matrigel can vary from batch to batch or change with prolonged storage (Aisenbrey and Murphy. Nat Rev Mater, 2020. 5(7): p. 539-551). In the second batch of Matrigel (Fig 5), the turning angles of both wild-type and TKO parasites are much lower than those of the corresponding lines measured earlier (Fig 4C). However, the differences in helicity (turning angle and dispersal ratio) remain highly significant between the mutant and the wild-type parent.

11. *As shown in their preceding publication, TLAP2 and TLAP3 bind to different regions of the cortical microtubules. While TLAP2 was shown to mostly localize across the full microtubule length except for a ring region at the apical end, TLP3 appeared to be only present at the apical end of cortical microtubules. How do the authors think TLAP2 and TLAP3 contribute to microtubule stability, also in combination with SPM1?*

Our previous work showed that TLAP2 and SPM1 together stabilize the portion of the microtubules distal to the apical section, which contains TLAP3. In the absence of TLAP2 and SPM1, TLAP3 stabilizes the apical section of the cortical microtubules.

This has been included in the introduction as background information to make the current manuscript more self-contained.
Line 87-90:
" We discovered that, in mature parasites, TLAP2 and SPM1 together stabilize the portion of the microtubules distal to the apical section, which contains TLAP3 [30]. In the absence of TLAP2 and SPM1, TLAP3 stabilizes the apical section of the cortical microtubules [30]."

12. *While the TKO has a very interesting phenotype considering motility patterns, the authors showed that depletion of the proteins does neither affect invasion nor egress of host cells. Hence, the authors might want to consider to change the order of their figures and put the current figure 3 as the final figure to hence end their publication with an intriguing finding in motility patterns upon microtubule disruption.*

Thanks the reviewer for the suggestion. We have in fact also considered this prior to the submission of the paper. We settled on the current order of figures, because the "negative" results, i.e. the lack of phenotype in host cell entry or egress, are quite surprising and interesting, given that we had expected the cortical microtubules should contribute to coping with the mechanical stress during entry and egress.

13. *As shown in their preceding paper, TLAP3 expression is established early on during daughter cell formation. In contrast, the authors show in the current paper that microtubules are only disorganized in the mother but not the daughter cells during replication. Do the authors have an idea why microtubule organization is only affected in mother cell but not the daughter cells even though all cells share the same cytoplasm.*

In fact, not only TLAP3 is incorporated in the daughter cytoskeleton early on, SPM1 (Tran et al. Eukaryotic Cell, 2012, 11(2): p. 206-16) and TLAP2 (new Figure S2) are also found in daughters. This apparent specific stabilizing activity of TLAP2, SPM1, and TLAP3 for mature cortical microtubules could be because daughter cortical microtubules acquire additional specific stabilizing factors.

This discussion is now included in Line 381-391:
"During division, the cortical microtubules of daughters grow continuously and do not display dynamic instability [33], indicating that these microtubules are stabilized by associated proteins. TLAP2, SPM1, and TLAP3 are recruited to the cortical microtubules of both mother and daughters [30, 31] (Fig S2), yet they have differential effects on the stability of these two sets of microtubules sharing the same cytoplasm. In the same TKO parasite, while the maternal microtubules are destabilized, the daughter microtubules grow normally. This apparent specific stabilizing activity of TLAP2, SPM1, and TLAP3 for mature cortical microtubules could be because daughter cortical microtubules acquire additional specific stabilizing factors. Of course, for those (hypothetical) factors, we would need to answer how they differentially act on the cortical microtubules of the mother and developing daughters, which coexist in the same cytoplasm. "

Figures:
- Figure 1, Panel C: Indicate clearly in the figure legend that the quantification in microtubule morphology refers to the mother cell but not the daughter cells.

This information has been added.
Line 779-780: "Quantification of the proportions of wild-type (top) and TKO (bottom) parasites that display various characteristics in the maternal cortical microtubule array at different stages"

- Figure 1: Have labels of subpanels aligned to the same side (TKO, Tub, IMC1,…)

Because each subpanel has a different layout, it is difficult to fit all the labels on the same side. We find the current arrangement adequate for readability.

- Figure 3, panel A: increase the distance between the lines to separate the parameters also visually.

This panel (now Figure 4A) has been revised as suggested.

- Figure 3, panel B: How does the motility paths look like for RHΔhx cold-treated? Provide a negative control here.

The motility paths for cold-treated *RHΔhx* parasites are now included in this panel (now Figure 4B). The quantification of net displacement, run length, run speed, turning angle, dispersal ratio and pitch for cold-treated *RHΔhx* and TKO parasites are also now included as part of the figure.

- Figure 4: Statistical tests are missing.

Statistical tests are now included.

- Figure 4, Panel B, D: The official scientific abbreviation for seconds is s not sec. Please change accordingly.

"Sec" has been replaced with "s".

- Figure 4, Panel D: Plot the median as part of the graph instead of having it written below the graph.

The median has been included as part of the graph.

- Figure 3, Figure 4: Exchange asterisks with exact p values.

Asterisks have been replaced with p values for these two figures (now Figure 4 and 6).

Reviewer 2

MAJOR COMMENTS:

1. *The data obtained from the 3D Matrigel are solid: TKO parasites partially lose helicity of the movement, following a "straighter" path during motility. The authors link this loss of helicity to the loss of the SPMTs but minimize the importance of alteration of parasite shape in this process. To firmly address if the absence of SPTMs is impacting or not on "banana-shape to the parasites, the authors should determine experimentally and quantitatively the parasite shape. This could be achieved by providing comparative morphological data on the TKO mutant and WT. Length, width and length:width ratio should be quantified as performed before (Leung et al. 2017) and ideally the curvature of the parasites should also be quantified. This would provide more robust data on the "banana-shape" of the parasites and assess if the TKO are "straighter" than WT parasites.*

We have now included in Table 1 the quantification of length, width, length:width ratio, as well as bowing (a parameter that is sensitive to the symmetry and curvature of the parasite) for the wild-type, *Δtlap2, Δtlap2Δspm1,* and TKO (*Δtlap2Δspm1Δtlap3*) parasites. We found that the TKO parasite, while still having a crescent shape, is significantly less bowed than all the other lines. We do not think the change in parasite shape is the likely explanation for the change in movement trajectory of the *Toxoplasma* microtubule mutants, because there does not appear to be a clear correlation between the parasite shape and linearity of movement. For example, the *Δtlap2Δspm1* mutant, which has a pronounced defect in the cortical microtubule array (Figure 2B), moves much more linearly than the wild-type parasite even though their bowing indices are similar (Figure 5,

Table 1).

2. *On the same 3D Matrigel experiment (Figure 3), the authors present an analysis of the motility for the TKO after cold treatment. A careful quantification of net displacement, run length, run speed, turning angle, dispersal ratio and pitch are required such that a proper comparison between TKO untreated and wild-type parasite can be achieved.*

Quantifications of net displacement, run length, run speed, turning angle, dispersal ratio and pitch for cold-treated TKO and wild-type parasites are included as part of the new Figure 4A. Similar to the untreated parasites, the helicity parameters (turning angle, dispersal ratio and pitch) are significantly different between the cold-treated wild-type and TKO parasite with the TKO parasite moving much more linearly than the wild-type parasite.

We note that while the net displacement, run length and run speed are similar in untreated parental and TKO parasites, these parameters are significantly higher for cold-treated wild-type parasites when compared with those for the cold-treated TKO. This difference is due almost entirely to a response to the cold-treatment by the wild-type parasite, and the lack of response by the TKO. This lack of response by the TKO parasite indicates that the residual microtubules in the TKO under untreated conditions do not have a notable impact on parasite motility. The increase in speed and persistence of wild-type parasite movement after cold- treatment is not due to a direct effect of the microtubules on motility because 1) the cold- treatment does not have a detectable effect on the microtubule array in the wild-type parasite, and 2) the untreated wild-type parasite, which has a full complement and normal arrangement of cortical microtubules, has the same speed and persistence as the TKO parasite with few microtubules and a disordered array.

Lastly, because outliers are trimmed from pooled data of all lines and conditions, with the addition of the cold-treated RH*Δhx* and TKO data, numbers for untreated RH*Δhx,* TKO, RH*Δku80* and *Δdcx* are slightly different from the original submission.

3. *The analysis of SPMTs morphology after cold treatment has been performed by IFA on intracellular parasites while the 3D motility experiments are performed with extracellular parasites. It would be relevant assess the morphology of their SPMTs after cold treatment (in WT and TKO) and quantify the different phenotypes (WT-like / short-sparse / absent) by IFA on extracellular parasites.*

Results for the analysis of the microtubule morphology in extracellular wild-type, TKO parasites, with and without cold-treatment, have been included in Figure 2A and B. The distribution of the different phenotypes is similar to what was observed for intracellular parasites.

4. *TKO parasites present dramatically shorten/absent SPMTs. This structure is not only important for parasite shape, but it has also been hypothesized that micronemes are positioned and trafficked along them (Leung et al. 2017). Furthermore, it is stated in the text that there is "lower incidence of entry attempts (…) for the TKO". It appears important to fully characterize the TKO mutant by performing quantitative microneme secretion-, host cell attachment- and invasion-assays.*

We have examined the amount of MIC2 secreted into the supernatant by extracellular RHΔhx parental and TKO parasites treated with the calcium ionophore A23187. We found that both lines respond robustly to the calcium ionophore treatment (Fig 6C and Fig S1). The TKO parasite on average secretes MIC2 at ~ 67% of the level of the RHΔhx parent (Fig S1), but the difference between the two lines is not statistically significant.

We have also completed the comparison of invasion efficiency between the wild-type and the TKO mutant using the immunofluorescence-based dual-color invasion assay, which shows that the TKO parasite invades at ~ 47% of the level of its wild-type parent (p= 0.026).

Re -Host cell attachment: We found that while the "invaded" part of the invasion assay is well-behaved and reproducible, the host cell attachment ("adhered but not invaded") is highly variable. Counting attached (but not invaded) parasites has not generated any meaningful results in our hands. This variability is believed to be a consequence of the parasites spending a relatively long

time sitting on and loosely attached to the cell surface before they start to penetrate, after which penetration is completed relatively quickly. During the adherent-but-not-yet-inside stage, the parasites are easily dislodged, so that the mechanical stress of the detergent extraction and washing that is necessary to develop the assay knocks them off. We have tried quite hard to standardize the washes and so forth to make the "adherent" counts more reproducible, but without success.

5. *Fig. 1C convincingly shows that daughter cell formation is not impaired in the TKO mutant. As stated in the manuscript, this could be due to other proteins stabilizing the nascent SPMTs. However, the role of SPM1, TLAP2 and TLAP3 (knocked out in the TKO) in daughter cell is not investigated. It is not clearly stated or shown if those three proteins are decorating the daughter cells SPMTs. The authors should provide this information by citing the literature or including the data on localization of the three proteins in daughter cells.*

All three proteins are recruited to the daughter cytoskeleton. We have now included the relevant citations for TLAP3 (*Liu et al. MBoC 2015. 27(3): p. 549-71*) and SPM1 (T*ran et al. Eukaryotic Cell, 2012, 11(2): p. 206-16*) as well as new data for TLAP2. Images of mE-TLAP2 knockin parasites are included as Figure S2, which demonstrates the association of TLAP2 with the daughter microtubules.

6. *There are other relevant mutants published that display altered shape/SPMTs morphologies (Ma et al. 2007 10.1091/mbc.e07-04-0379 or Liu et al. 2016). These parasites either harbor either a mutation in the alpha-tubulin gene or overexpress TLAP2 and in both cases exhibit longer/straighter SPMTs that lead to longer and straighter parasites. A comparison of the trajectories of these mutants with TKO would be very complementary. Inclusion of these mutant parasites in the 3D Matrigel analysis reported here would reinforce the claims and give more weight to the work.*

We did not show in our previous paper (*Liu et al. 2015*) that longer and straighter microtubules lead to longer straighter parasites. Gross shape change in cells overexpressing TLAP2 is likely due to aberrant cell division, but not a direct result from changes in the cortical microtubules.

Ma et al. 2007 did not show that longer and straighter microtubules lead to longer straighter parasites, also. In that paper, one image was included to show that the microtubules in one alpha-tubulin H28Q parasite were longer than one wild-type parasite. However, there was no quantitation of the correlation of microtubule length/straightness with parasite shape. This mutant also has replication defects, which affects parasite shape indirectly. Therefore, although alpha-tubulin H28Q is an interesting mutant, available evidence is insufficient to justify the suggested experiment. Further characterization of that mutant is beyond the scope of the current work.

MINOR COMMENTS:

- Some sentences are convoluted and would benefit to be shortened.

We have parsed some of the long sentences into shorter ones. Please see below for response to individual comments.

- Figure 2 could be a supplementary figure. The pipeline used in this study for trajectory tracking is excellent but similar protocol have been published before (Leung et al. 2014).

The main point for this figure is to demonstrate how the DIC images are converted to images with high contrast (similar to fluorescence images). This conversion is essential for automated determination of cell positions and required to render DIC-based data amenable to 3-D cell tracking. This was not reported in *Leung et al. 2014* (*PLoS One*) which used fluorescence images for tracking cell movement. We therefore think that it is of value to report the image processing protocol as a main figure.

- The abstract is too long. Journal of Cell Science guidelines indicate that the abstract should be

no more than 180 words.

Abstract has now been shortened to be within the 180-word limit.

Include statistics for graph in Fig. 4B and 4D.

Statistical tests are now included for this figure (now Figure 6).

- Fig. 4B represents "time taken to disperse" but this is not what is described in the results section. In the text it is mentioned egress, not time to disperse.

“time taken to disperse” is now mentioned in the text (Line 314)

- Line 1: Toxoplasma gondii in full. Toxoplasma gondii is now written in full.

- Line 31: Correct "spirally"

The typo has been corrected.

- Line 35: please rephrase the sentence “In wild-type parasites, the cortical microtubules in mature parasites (…)” so it is not repetitive or simply delete "in wild-type parasites".

This sentence has been deleted due to the shortening of the abstract.

- Line 53-55: the sentence is difficult to understand. What is the difference between "host cell entry" and "infection" ?

This sentence has been deleted due to the shortening of the abstract.

- Line 65-69: the sentence is too long.

This sentence has now been separated into two as follows (Line 49-53):
"As obligate intracellular organisms, a successful infection requires the parasite to travel over a long distance through tissues with varying biochemical and biophysical properties [3] as well as to enter into host cells. The latter requires a shorter travel distance, but perhaps more finesse, as the host cell plasma membrane needs to remain intact to allow the parasite to use host cell resources for proliferation."

- Line 66-67: "successful infection requires the parasite to travel over a long distance through tissues". If this is the case and it has been shown previously, a citation is needed.

A reference has been added. Please see above.

- Line 72-77: the sentence is too long. To be split to separate the citations and refer to them more precisely with explanatory sentences.

This sentence has been split into two with separate citations.
Line 56-60: “Chemical inhibition as well as genetic manipulations showed that actin and a series of apical and cortical myosin motors provide the underlying force for the parasite motility [5-9]. The current model posits that the internal force is transmitted to the parasite surface through a coupling between the actomyosin complex and the secreted transmembrane adhesins [10]. ”

- Line 78: “energy is injected into the system” to be rephrased and explained.

This sentence has now been changed to (Line 60-61) “The characterization of these factors has significantly advanced the understanding of parasite motility [5-9, 11-15].”

- Line 80: “polymerization is likely to occur dynamically”, please include citation as extensive

work has been done on intrinsic instability of apicomplexan F-actin.

Citations have been included.
Line 61-63: "However, while actin polymerization is critical for parasite movement, no native actin filaments have been detected, suggesting that the polymerization is likely to occur dynamically [16-21]."

- Line 93: "helical, or more accurately," is dispensable. Consider referring to "spiral pattern".

The sentence has been revised as suggested.
Line 76: "... 22 cortical microtubules that are arranged in a spiral pattern"

Reviewer 3

Minor comments:
1)Motility in cold-treated parasites: On line 167 authors state that the cortical MT arrays are significantly restored after 20 minutes of temperature shift from 4C to 37C. The authors then go on to image motility in cold treated parasites at 37C (paragraph beginning on line 210). The authors state the imaging is done "immediately" after shift to 37C but it is unclear how long the imaging takes and if the MTs remain disrupted for the entire imaging period.

Image acquisition for 3-D motility was started within ~2-3 mins after the suspension had entered the 37°C chamber.

More details for the setting up the 3D motility assay are now included in Methods. Line 603-609: "For cold-treated parasites, cultures with intracellular parasites were placed at ~ 7ºC for 2 hr, before harvesting as described in [51]. The harvested parasite suspension was kept on ice for ~30 sec, then mixed with an equal volume of Matrigel (Corning, 356237) on ice and pipetted into the pre-heated flow chamber. The chamber was then sealed with a polymerizable liquid silicone rubber (Nobilium Inc, Nobilsil #7453+7434) and image acquisition was started within ~2-3 mins after the suspension had entered the 37°C chamber."

2)The authors have previously demonstrated that micronemes are associated with the cortical MTs, Leung et al 2017, PMID: 28331073. One would anticipate that the disruption to the microtubules would also lead to a chance in the localization of micronemes, however microneme secretion does not appear to be affected as parasite invasion was not disrupted. Did the authors evaluate the localization of micronemes to determine if (a) MTs are needed for apical localization of the micronemes and (b) if apical localization of micronemes is needed for secretion.

We have determined the localization of micronemes by immunofluorescence using an anti-MIC2 antibody in the TKO and the wild-type parasite (Fig 6A-B). There is a significant difference in the distribution of microneme vesicles in the TKO vs the wild-type parasite. In ~40% of the untreated TKO parasite and ~2% of the wild-type parasite, microneme vesicles are not concentrated in the apical cap region. When cold-treated for 2 hr, this percentage increases to ~67% (±3.5% SEM) for the TKO parasite and ~ 10% for the wild-type parasite. In parasites that lose the microneme enrichment at the apical cap, an intense concentration of MIC2 labeling remains at the parasite apex (Fig 6A).

We also examined the amount of MIC2 secreted into the supernatant by extracellular RHΔhx parental and TKO parasites treated with the calcium ionophore A23187. We found that both lines respond robustly to the calcium ionophore treatment (Fig 6C and Fig S1). The TKO parasite on average secretes MIC2 at ~ 67% of the level of the RHΔhx parent (Fig S1), but the difference between the two lines is not statistically significant.

Second decision letter

MS ID#: JOCES/2023/261270

MS TITLE: The cortical microtubules of *Toxoplasma gondii* underlie the helicity of parasite movement

AUTHORS: Isadonna F. Tengganu, Luisa F. Arias Padilla, Jonathan Munera Lopez, Jun Liu, Peter T Brown, John M Murray, and Ke Hu
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave very favourable reports but raised some minor text/fig corrections that are easier to fix at this stage rather than the proof stage. I hope that you will be able to carry these out because I would like to be able to accept your paper once they are done.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

see my previous report

Comments for the author

This is a well done revision that has in my view improved the manuscript. There are now only a few minor issues still e.g. missing address nr 3, some cited literature is not in journal format that the authors should address.

Reviewer 2

Advance summary and potential significance to field

The authors have addressed all the concerns and shortcomings and the manuscript is suitable for publication

Comments for the author

The panel C of figure 6 included in the revsion is lacking the labeling of the parasite strains analyzed

Reviewer 3

Advance summary and potential significance to field

The authors have done an excellent job addressing reviewer concerns and revising the manuscript. I have no further concerns.

Comments for the author

n/a

Second revision

Author response to reviewers' comments

Reviewer 1
- There are now only a few minor issues still e.g. missing address nr 3, some cited literature is not in journal format that the authors should address.

The address for number 3 on the title page was included in the same line as that for number 2 in the previous versions of the manuscript. Now it is listed as a separate line (Line 10).

The citations have been reformatted using Harvard referencing.

Reviewer 2
- The panel C of figure 6 included in the revsion is lacking the labeling of the parasite strains analyzed

We thank the reviewer for noticing that the labels for parasite strains were missing. The parasite strains are now labeled in Figure 6C and Figure S1.

Third decision letter

MS ID#: JOCES/2023/261270

MS TITLE: The cortical microtubules of *Toxoplasma gondii* underlie the helicity of parasite movement

AUTHORS: Isadonna F. Tengganu, Luisa F. Arias Padilla, Jonathan Munera Lopez, Jun Liu, Peter T Brown, John M Murray, and Ke Hu
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.