γ-tubulin complex controls the nucleation of tubulin-based structures in Apicomplexa

Romuald Haase, Annet Puthenpurackal, Bohumil Maco, Amandine Guérin, and Dominique Soldati

Corresponding author(s): Dominique Soldati, Faculty of Medicine - University of Geneva

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E24-03-0100
TITLE: γ-tubulin complex controls the nucleation of tubulin-based structures in Apicomplexa

Dear Dr. Soldati:

Your manuscript has been reviewed by 2 experts in the field of microtubule machineries in Apicomplexa, who has greatly appreciated your work.
One reviewer requests some clarifications about the phenotype of gamma tubulin-deficient parasites, and both reviews raise minor points for editorial improvement of your text.
Thank you very much for your revisions.

Sincerely,

Isabelle Coppens
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Soldati,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

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Thank you for submitting your manuscript to MBoC. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

γ-tubulin complex controls the nucleation of tubulin-based structures in Apicomplexa
Romuald Haase, Annet Puthenpurackal, Bohumil Maco, Amandine Guérin and Dominique Soldati-Favre

Overall significance: This paper describes the role of the γ-tubulin ring complex in two apicomplexan parasites, Toxoplasma gondii and Cryptosporidium parvum. Loss of this complex by auxin-induced degradation impairs nucleation of the microtubules that form the spindle, conoid and subpellicular microtubules, in line with the role of this complex in a wide variety of other eukaryotes. The data presented here, particularly the quality of the images, is quite high and is of interest to cell biologists.

Specific points:
Line 59: could cite Wang et al paper, PMID 34576816.
Line 60-61: "Gamma tubulin (γ-tubulin) is a highly conserved protein across eukaryotic species, required for microtubule nucleation (Oakley, Paolillo, and Zheng 2015)." I would suggest that this be extended to state that it is a universal and essential protein in all eukaryotes.
Line 67-70: "Interestingly, both Toxoplasma and Cryptosporidium possess essential tubulin-based structures such as the centrioles, conoid tubulin fibers, subpellicular microtubules (SPMTs) and intraconoidal microtubules (ICMTs) (for T. gondii) whose origins remain unclear (Dos Santos Pacheco et al. 2020)." This sentence is unclear and hard to read. How about: "Both Toxoplasma and Cryptosporidium possess essential tubulin-based structures whose origins remain unclear (Dos Santos Pacheco et al. 2020)." Talk about specific examples later, separately.
Line 100: "undividing" should be "non-dividing"
Line 101: "no signal is detected" - Figure 1B extracellular has a signal and this is discussed later so your statement needs to be re-written to be consistent with the data.
Line 109-110: "In T. gondii, the centrosome architecture consists of an outer core (distal) and inner core (proximal)." Add citations for this work.
Line 110-112: "The centrioles, along with centrin1 protein, are part of the outer core. Colocalization with Centrin1 shows a shifted γ-tubulin staining suggesting its localization in the inner core of the centrosome as previously reported (Suvorova et al. 2015) (Fig1C)." This is worded in a confusing way because the word co-localization suggests that the proteins are colocalized (together). How about "Dual labeling of γ-tubulin and centrin1 suggests that it localizes to the inner core of the centrosome as previously reported (Suvorova et al. 2015)."
Line 118: "(Supplemental Fig. 2A)." should be (Supplemental Fig. 2B).
Line 136: "denoted by" should be "defined by"
Line 143: "split" should be "divide"
Line 212-14: "Interestingly, γ-tubulin protein is absent in extracellular sporozoites and in intracellular parasites harboring 1 nucleus contradicting a previous report using anti-γ-tubulin antibody (Wang et al. 2024)(Fig4F) (Supplemental Fig3B)." I suggest changing the wording to "undetectable" rather than absent.
Line 217: (Fig4F) should be (Fig4G)
Line 219: (Fig4F) should be (Fig4G)
Line 233-35: "Interestingly, the association of γ-tubulin with the forming apical complex appears to be very transient, always on the opposite side as the tubulin staining suggesting a role its role in the initiation but not for elongation of microtubules." This could be expanded upon. Since the conoid and subpellicular microtubules are not dynamic microtubules in the same way that spindle microtubules are, this is not surprising.
Line 235: "tubulin staining suggesting a role its role in the" typo/grammar
Line 244-46: "The origin and nucleation process of these abnormal microtubules, which appeared to be γ-tubulin-independent, remain open questions, warranting further investigation." This could be expanded on. Presumably, protein synthesis, including synthesis of a-b tubulin heterodimers continues in the absence of cell division. The abnormally long microtubules that emerge from the centrioles may be simply due to polymerization of newly synthesized tubulin to maintain the critical concentration.
Supplemental figure 2A: the word "parasites" on the axis is spelled incorrectly
I strongly suggest that supplemental figure 2 C and D be integrated as a 5th figure in the paper body.

Reviewer #2 (Remarks to the Author):

The manuscript by Haase and colleagues explores the role of γ-tubulin and Gamma Tubulin Complex proteins (GCPs) in the nucleation of tubulin-based structures during Toxoplasma gondii's cell division. The study also describes the location of γ-tubulin in Cryptosporidium. They use expansion microscopy combined with conditional knockout for the fonctional analysis done in

Toxoplasma.
The authors convincingly showed the presence of γ-tubulin at the spindle poles during mitosis in both models and its association to nascent apical complexes in daughter tachyzoites. Loss of γ-tubulin induced strong morphological defects, including impairement in nuclear scission and the formation of abnormally long microtubules. The authors conclude into an absence of duplication of centrioles, loss of conoid, of spindle microtubule formation and subpellicular microtubule nucleation.
While the location of the γ-tubulin complex is convincing, some of the interpretations of the phenotype of the mutants would require more analysis, as explained in the major comments. The captions on the images are really sketchy for a non specialist of division and cytoskeleton structures of Toxoplasma and Cryptosporidium.

Major Comments:

1) One main comment concerns the phenotype of γ-tubulin-depleted parasites. Based on figure 2D (first panel U-ExM), the authors conclude that γ-tubulin-depleted parasites do not duplicate centriole. However, in figure 2C (panel with centrin 1 IFA), the vacuole shows several dots of centrin 1. This is an important discrepancy which deserves to be addressed. One might then wonder whether there are really no centrioles duplication in the mutant. A colocalization with centrin 1 in U-ExM will shed some light on this. Similarly, there are no staining of the mutant with conoid marker or spindle microtubules (EB1) to support a role in initiation of these structures. At 18h auxin treatment, we expect to see no or only the initial conoid of the mother. These stainings would be more relevant than microneme or rhoptries staining.
The staining with EB1 may also help to understand the nature of these mysterious long microtubules in the mutant, in particular if they correspond to abnormal spindle microtubules.

2) A second question is raised by the presence of a γ-tubulin punctate signal in 40% of extracellular parasites. If γ-tubulin is, as suggested by its localization only in early stages of intracellular duplication, associated with microtubules inititiation and then diffuses in the cytosol, how 40 % of extracellular parasites could have initiated division? In fig. 1D, the parasite has not yet duplicated its pair of centrioles, and a single punctum of γ-tubulin between the two centrioles is detected at this stage, showing that γ-tubulin expression starts before duplication. Since this is consistent with a role in centriole duplication, this should be more clearly stated.
In Figure 1B, a colocalisation with centrin 1 to discriminate between dividing and undividing parasites is necessary. A quantification of the different stages on intracellular parasites (with the costaining with centrin 1), will be more informative than in extracellular.

Minor comments :

1) The text in figure 1A and the structures of the apical complex are too small. A zoom for each stage, showing the centrioles, conoid, ICMTs... would help non expert readers.
2) The colocalization with centrin 1 shows a shifted location, suggesting that γ-tubulin is part of inner core of centrosome, and the authors said that a previous study associated γ-tubulin to inner core. However, in their study Sururova et al claimed that γ-tubulin is at the outer core. This discrepancy should be discussed
3) In U-ExM (Figure 1D, 1E, 1F) labeling by arrows the centrioles, the conoid, the nascent SPMTs, or a cartoon with the corresponding structures, would greatly help understanding the figures.
4) The timing of auxin depletion in Figure 2D is not indicated in figure or legend, thus precluding to know how many divisions should have occurred in control parasites.
5) Indicate in the legend what VVL specifically stains in Cryptosporidium.
6) Discussion : lines 228-230, this study does not demonstrate a conserved role and localization of the γ-tubulin in Toxoplasma and Cryptosporidium. The conservation is only partial since the role of the protein has not been addressed in Cryptosporidium and the γ-tubulin was not detected in the forming cytoskeleton of Cryptosporidium merozoites. The sentence must be rephrased.
7) Fig 4G is not mentioned in the text.
8) In Fig4 B, the label « centrosome » corresponding to centrin detection, is correct, but the caption "centrioles" underneath is an overstatement since these organelles have not been observed so far in Cryptosporidium (such a structure is frequently absent in asexual stages of Apicomplexa, such as in Plasmodium sp. for ex).

Reviewer #1

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Specific points:

Line 59: could cite Wang et al paper, PMID 34576816.

The reference has been added.

Line 60-61: "Gamma tubulin (γ-tubulin) is a highly conserved protein across eukaryotic species, required for microtubule nucleation (Oakley, Paolillo, and Zheng 2015)." I would suggest that this be extended to state that it is a universal and essential protein in all eukaryotes.

The sentence has been rephrased as suggested.

Line 67-70: "Interestingly, both Toxoplasma and Cryptosporidium possess essential tubulin-based structures such as the centrioles, conoid tubulin fibers, subpellicular microtubules (SPMTs) and intraconoidal microtubules (ICMTs) (for T. gondii) whose origins remain unclear (Dos Santos Pacheco et al. 2020)." This sentence is unclear and hard to read. How about: "Both Toxoplasma and Cryptosporidium possess essential tubulin-based structures whose origins remain unclear (Dos Santos Pacheco et al. 2020)." Talk about specific examples later, separately.

The sentence has been rephrased as proposed.

Line 100: "undividing" should be "non-dividing"

This is corrected.

Line 101: "no signal is detected" - Figure 1B extracellular has a signal and this is discussed later so your statement needs to be re-written to be consistent with the data.

This has been rephrased with reference to the data displayed in Fig1B (now Fig2A**).**

Line 109-110: "In T. gondii, the centrosome architecture consists of an outer core (distal) and inner core (proximal)." Add citations for this work.

This section has been expended and the Suvorova et al. 2015 has been included.

Line 110-112: "The centrioles, along with centrin1 protein, are part of the outer core. Colocalization with Centrin1 shows a shifted γ-tubulin staining suggesting its localization in the inner core of the centrosome as previously reported (Suvorova et al. 2015) (Fig1C)." This is worded in a confusing way because the word co-localization suggests that the proteins are colocalized (together). How about "Dual labeling of γ-tubulin and centrin1 suggests

that it localizes to the inner core of the centrosome as previously reported (Suvorova et al. 2015)."
The text has been edited as requested. The term “colocalization” has been replaced by “dual labeling”

Line 118: "(Supplemental Fig. 2A)." should be (Supplemental Fig. 2B).
This has been fixed and it is now referencing to Supplementary Fig 2C.

Line 136: "denoted by" should be "defined by"
Done.

Line 143: "split" should be "divide"
Done.

Line 212-14: "Interestingly, γ-tubulin protein is absent in extracellular sporozoites and in intracellular parasites harboring 1 nucleus contradicting a previous report using anti-γ-tubulin antibody (Wang et al. 2024)(Fig4F) (Supplemental Fig3B)." I suggest changing the wording to "undetectable" rather than absent.
“absent” has been replaced by “undetectable”.

Line 217: (Fig4F) should be (Fig4G)
This has been corrected and it corresponds now to Fig 6D.

Line 219: (Fig4F) should be (Fig4G)
This has been corrected and corresponds now to Fig 6D and supplementary Fig 3A

Line 233-35: "Interestingly, the association of γ-tubulin with the forming apical complex appears to be very transient, always on the opposite side as the tubulin staining suggesting a role its role in the initiation but not for elongation of microtubules." This could be expanded upon. Since the conoid and subpellicular microtubules are not dynamic microtubules in the same way that spindle microtubules are, this is not surprising.
We concur with the reviewer that the transient association of γ-tubulin was expected. We have removed the word ‘Interestingly’ and expanded the sentence.

Line 235: "tubulin staining suggesting a role its role in the" typo/grammar
Corrected

Line 244-46: "The origin and nucleation process of these abnormal microtubules, which appeared to be γ-tubulin-independent, remain open questions, warranting further investigation." This could be expanded on. Presumably, protein synthesis, including synthesis of a-b tubulin heterodimers continues in the absence of cell division. The abnormally long microtubules that emerge from the centrioles may be simply due to polymerization of newly synthesized tubulin to maintain the critical concentration.
Supplemental figure 2A: the word "parasites" on the axis is spelled incorrectly

I strongly suggest that supplemental figure 2 C and D be integrated as a 5th figure in the paper body.
As recommended the data of supplementary figure 2A has been moved to a main figure. However, to keep the flow of the manuscript, the data is included in the new figure 4.

Reviewer #2

The manuscript by Haase and colleagues explores the role of γ-tubulin and Gamma Tubulin Complex proteins (GCPs) in the nucleation of tubulin-based structures during Toxoplasma gondii's cell division. The study also describes the location of γ-tubulin in Cryptosporidium. They use expansion microscopy combined with conditional knockout for the fonctional analysis done in Toxoplasma.
The authors convincingly showed the presence of γ-tubulin at the spindle poles during mitosis in both models and its association to nascent apical complexes in daughter tachyzoites. Loss of γ-tubulin induced strong morphological defects, including impairement in nuclear scission and the formation of abnormally long microtubules. The authors conclude into an absence of duplication of centrioles, loss of conoid, of spindle microtubule formation and subpellicular microtubule nucleation.
While the location of the γ-tubulin complex is convincing, some of the interpretations of the phenotype of the mutants would require more analysis, as explained in the major comments. The captions on the images are really sketchy for a non specialist of division and cytoskeleton structures of Toxoplasma and Cryptosporidium.

Major Comments:
1) One main comment concerns the phenotype of γ-tubulin-depleted parasites. Based on figure 2D (first panel U-ExM), the authors conclude that γ-tubulin-depleted parasites do not duplicate centriole. However, in figure 2C (panel with centrin 1 IFA), the vacuole shows several dots of centrin 1. This is an important discrepancy which deserves to be addressed. One might then wonder whether there are really no centrioles duplication in the mutant. A colocalization with centrin 1 in U-ExM will shed some light on this. Similarly, there are no staining of the mutant with conoid marker or spindle microtubules (EB1) to support a role in initiation of these structures. At 18h auxin treatment, we expect to see no or only the initial conoid of the mother. These stainings would be more relevant than microneme or rhoptries staining.
The staining with EB1 may also help to understand the nature of these mysterious long microtubules in the mutant, in particular if they correspond to abnormal spindle microtubules.

We appreciate the suggestion to clarify phenotypes requiring further explanation. Regarding the capacity of the γ-tubulin-depleted parasite to duplicate their centrioles, we believe, as explained in the main text, that the parasites cannot duplicate their centrioles (data in Figure 3D / previously 2D). The multiple dots of centrin1 observed in Figure 3C (previously 3D) likely result from protein fragmentation rather than centriole duplication. To confirm our

hypothesis, we performed U-ExM using centrin1 in γ-tubulin-depleted parasites as suggested. The new data, presented in Supplementary Fig2E, corroborates our previous observations by classical IFA (Fig3C), showing fragmented centrin1 staining without any associated tubulin structure.

To investigate the nature of these mysterious long microtubules, we employed two complementary approaches: tagging and leveraging the properties of SPMTs. We utilized the polyglutamylation properties of *T. gondii* SPMTs. In γ-tubulin-depleted parasites, these abnormal microtubules were decorated with PolyE as presented in the new figure 4. Additionally, IMC1 attachment was observed on these microtubules under U-ExM. These observations suggest that these microtubules could be SPMTs. To test this hypothesis, we tagged four microtubule-associated proteins—ICMAP2 (intraconoidal microtubules), DCX (conoid fibers), SPM1 (subpellicular microtubules), and EB1 (spindle microtubules)—in the γ-tubulin-mAID-HA background. In γ-tubulin-depleted parasites, none of these proteins localized on the microtubules, as shown in Fig4B. The fact that none of these proteins, especially SPM1 (which is found in the lumen of SPMTs), localized on these microtubules suggests that their nature is undefined.

2) A second question is raised by the presence of a γ-tubulin punctate signal in 40% of extracellular parasites. If γ-tubulin is, as suggested by its localization only in early stages of intracellular duplication, associated with microtubules inititiation and then diffuses in the cytosol, how 40 % of extracellular parasites could have initiated division? In fig. 1D, the parasite has not yet duplicated its pair of centrioles, and a single punctum of γ-tubulin between the two centrioles is detected at this stage, showing that γ-tubulin expression starts before duplication. Since this is consistent with a role in centriole duplication, this should be more clearly stated.

In Figure 1B, a colocalisation with centrin 1 to discriminate between dividing and undividing parasites is necessary. A quantification of the different stages on intracellular parasites (with the costaining with centrin 1), will be more informative than in extracellular.

In our view, centrin1 is not a cellular marker distinguishing dividing from non-dividing parasites but rather tracks centriole duplication. To further support our observations, we have extended our analysis of γ-tubulin in relation to centrin1 from extracellular to intracellular parasites. We observed that in the 40% of extracellular parasites displaying a punctate γ-tubulin signal, this staining appears as a globular signal between the centrioles (now shown in Fig2B and Supplementary Fig2A).

Similarly, in intracellular parasites before centriole duplication, γ-tubulin is located between the centrioles. After centriole duplication, γ-tubulin is observed on both sides of the early spindle microtubules and surrounding the centrioles. The presence of a punctate γ-tubulin signal in some extracellular parasites remains an open question, but it could potentially represent a "pre-loading mechanism," where parasites pre-load γ-tubulin in the centrosomal region to initiate endodyogeny immediately upon invasion.

Additionally, we performed an IFA with another early cell division marker, Pcr4 (now in Supplementary Fig2B). In extracellular parasites, Pcr4 was detected only apically, without additional staining corresponding to conoid formation, indicating no active endodyogeny at this stage, as expected.

Minor comments :

1) The text in figure 1A and the structures of the apical complex are too small. A zoom for each stage, showing the centrioles, conoid, ICMTs... would help non expert readers.

The scheme has been separated and is now displayed in Figure1. It has been remodeled accordingly and zoom on relevant area are shown for clarification.

2) The colocalization with centrin 1 shows a shifted location, suggesting that γ-tubulin is part of inner core of centrosome, and the authors said that a previous study associated γ-tubulin to inner core. However, in their study Sururova et al claimed that γ-tubulin is at the outer core. This discrepancy should be discussed

It has been corrected. Both centrin 1 and γ-tubulin are at the outer core of the centrosome although slightly shifted.

3) In U-ExM (Figure 1D, 1E, 1F) labeling by arrows the centrioles, the conoid, the nascent SPMTs, or a cartoon with the corresponding structures, would greatly help understanding the figures.

Labelings on the corresponding panel have been added to help understand the figures.

4) The timing of auxin depletion in Figure 2D is not indicated in figure or legend, thus precluding to know how many divisions should have occurred in control parasites.

Parasites were treated for 12 hours. However, due to the asynchronous nature of invasion, different stages of spark microtubules were imaged within the same treatment period. The treatment duration has been noted in the figure legend.

5) Indicate in the legend what VVL specifically stains in Cryptosporidium.
This has been added in the legend as suggested.

6) Discussion : lines 228-230, this study does not demonstrate a conserved role and localization of the γ-tubulin in Toxoplasma and Cryptosporidium. The conservation is only partial since the role of the protein has not been addressed in Cryptosporidium and the γ-tubulin was not detected in the forming cytoskeleton of Cryptosporidium merozoites. The sentence must be rephrased.

Thank you for pointing it out. The conclusion in the discussion session has been rephrased to reflect the data shown.

7) Fig 4G is not mentioned in the text.
This has been corrected, it now corresponds to Figure 6G.

8) In Fig4 B, the label « centrosome » corresponding to centrin detection, is correct, but the caption "centrioles" underneath is an overstatement since these organelles have not been observed so far in Cryptosporidium (such a structure is frequently absent in asexual stages of Apicomplexa, such as in Plasmodium sp. for ex).
It has been modified as suggested (now Figure 6B).

RE: Manuscript #E24-03-0100R
TITLE: "γ-tubulin complex controls the nucleation of tubulin-based structures in Apicomplexa"

Dear Dr. Soldati:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

We recommend this manuscript to be published in this journal. Excellent story!

Sincerely,
Isabelle Coppens
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Soldati:

Congratulations on the acceptance of your manuscript! Thank you for publishing your work in Molecular Biology of the Cell (MBoC).

Within 10 days, an unedited PDF of your manuscript will be published on MBoC in Press, an early-release journal version. The date your manuscript appears on this site is the official publication date.

Your copyedited and typeset manuscript will be scheduled for publication in the next available issue of MBoC. Once your paper is ready for review, our production team will notify you. In the summer of 2024, our production provider will introduce an online proofing solution, which will streamline the process of checking and validating author changes. This will result in improved typesetting quality, enhanced digital content, and a faster overall process.

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We look forward to publishing your paper in MBoC.
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Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

My review comments have been adequately addressed.

Reviewer #2 (Remarks to the Author):

The authors have addressed my comments satisfactorily. I look forward to seeing this work published.