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Toxoplasma gondii myosin F, an essential motor for centrosomes positioning and apicoplast inheritance

Damien Jacot, Wassim Daher and Dominique Soldati-Favre

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Transaction Report:

(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.)

Editor: David del Alamo

1st Editorial Decision 07 February 2013

Thank you for the submission of your manuscript entitled "Toxoplasma gondii myosin F is a class XXII motor essential for apicoplast inheritance" and please accept my apologies for the delay in the decision, due to the recent holiday season. We have now received the full set of reports from the referees that were asked to evaluate your study, which I copy below. As all four referees believe in the high interest of your manuscript and their general comments are positive, I would like to invite you to revise it.

Without going into details that you will find below, referees #1, #2 and #4 consider that your manuscript should be published in The EMBO Journal and propose a number of points for you to consider -most of them rather minor- in order to improve your message before final acceptance can be granted. As you will also see, referee #3 is quite less enthusiastic about your work and raises a number of queries with the aim of elucidating the mechanistic details of the role of myoF in apicoplast inheritance. While we agree that including this kind of mechanistic insight would certainly increase the interest and potential reach of your study, we also believe that it would be out of the scope of this manuscript for you to embark on such experimental work. Naturally, if you already have data at hand that could address some of the concerns of referee #3, it would be only in your best interest to include them, although this information will not be determinant for the acceptance of your manuscript.

Note that referee #4 has included a PDF file of your manuscript with some hand-made annotations suggesting some other minor changes. Due to technical limitations, I cannot send you this file as an

attachment in my decision letter, but it will be included in a second e-mail instead. I apologize for the inconvenience.

Please be aware that it is 'The EMBO Journal' policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will essentially depend on the completeness of your responses included in the next version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process iniciative, please visit our website: http://www.nature.com/emboj/about/process.html

Once more, do not hesitate to contact me by e-mail or on the phone in case you have any questions.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS:

Referee #1 (General Remarks):

A role for an actomyosin motor and apicoplast inheritance is described. Actin inhibitor cytocholasin D is shown to perturb apicoplast inheritance in Toxoplasma gondii, Plasmodium falciparum, P. berghei. Knockdowns of profilin, actin depolymerization factor and a forming also perturbed apicoplast inheritance. A specific myosin (MyoF) is shown to accumulate near apicoplasts during division. Two knockdowns of MyoF were undertaken: a knock-in of a regulatable promoter, and expression of a dominant negative dysfunctional competitor MyoF. Both knockdowns exhibit perturbation of apicoplast inheritance. The experiments are well designed, clearly presented and the interpretations are valid. This work demonstrates an important new role for actomyosin in the accurate segregation of daughter organelles into daughter cells in the Phylum Apicomplexa. Whether or not such a system operates in the plastids of algae and plants, or is restricted to Phylum Apicomplexa with its bizarre cytokinesis, is not discussed.

I have made various corrections, suggestions and queries directly on a hard copy of the manuscript and a PDF has been sent to the editor and should be supplied to the authors.

I also suggest a number of changes to clarify things for the reader.

Mitochondria are shown in some parts of Fig 1 but not all. As it stands, one might infer that mitochondria disappear and are generated de novo after endodyogeny is completed, which is not the case. Fix this.

Throughout. The formal name of this group of parasites is Phylum Apicomplexa. The authors use the name 'Apicomplexan' in places. Strictly speaking, apicomplexan is an adjective (i.e. apicomplexan parasite) not a name and shouldn't be capitalized.
Fig2B (and elsewhere). Other users of the Cpn60 processing assay to analyze apicoplast defects have identified the lower mass band as mitochondrial Cpn60 (Agrawal JBC 2009). Do the authors disagree with this interpretation? Why?

Fig 2E. The minus cytocholasin D parasite(s) look(s) to have already egressed, but the lack of a differential interference contrast image makes it hard to know. In the Fig 2E panel where the effect of the addition of cytocholasin D is shown a brightfield image is supplied for this schizont. Be consistent. Show bright field images for both and preferably the same stage.

Page 9. What system of nomenclature is being used for genes versus proteins? I deduce that the gene name is in italics and the protein in non-italic font. Even so, the sentences sometimes appear to be referring to a gene when they are talking about a protein and vice versa.

Throughout. The 'w' in western blot should only be capitalized when used at the beginning of a sentence. Although convention allows capitalization, it is preferable to perpetuate the commemoration of Edwin Southern by restricting capitalization to the proper noun - Southern blotting.

Figure 5 - The line pointing to pCpn60 is too high. It doesn't point to the level of the band.

Figure 7 - It is confusing to have four images and two figure labels (7A & 7B). Change to 7A-C. This occurs in many places in the figures. I appreciate that this is to keep subgroups simple within a compound figure, but it can be ambiguous. I would recommend that every image has a number/letter designation, but that's up to the editor.

Page 14 - Why do the authors refer to 'mitochondrial content'? I appreciate that the marker (HSP70) is for a soluble matrix protein, but other markers used are similar and the location of the entire organelle is inferred from these markers.

I'm not a big fan of the 'horizontal' and 'vertical' terminology (page 13) for the orientation of the daughter cells during endodyogeny. I read this many times, but it just isn't intuitive. Would a clock face analogy work better? For instance, 12 o'clock for wild type, 6 o'clock, and occasional 9/3 o'clock for DDMyoF expressing parasites.
Fig 9B & 9C - Again the mitochondria disappear and magically reappear after endodyogeny. Fix this.

The Discussion needs to incorporate the connector between the apicoplast and centrosome (Francia PLoS Biol 2012).

Referee #2 (General Remarks):

This paper by Jacot et al presents evidence for the role of the actomyosin cytosekeleton in the inheritance of the apicoplast - a relict non-photosynthetic plastid in the phylum Apicomplexa. In particular the authors present a role for a conserved class XXII myosin - MyoF in controlling apicoplast segregation. The authors primarily use Toxoplasma gondii to study this phenomenon but also show the applicability of their findings, where possible to the malaria-causing parasite Plasmodium spp.

This study will have a wide readership from scientists interested in either apicomplexan or myosin biology and therefore is appropriate for a EMBO Journal.

This study is well executed and is well controlled and their conclusions are largely warranted by the data presented. However, I believe a few issues should be addressed before its publication.

Major points:

1. The authors have focussed the role of MyoF and actin dynamics in apicoplast segregation yet it is clear that there is a role for this system in the positioning of the rhoptries. This is mostly glossed over although presented and mentioned as work submitted in another paper. Is there any strong justification for leaving this work in? I think it detracts a from the central message of the paper.

2. Figure S2 (discussed on p8): It is not clear to me why this section is not presented in the main text. It is also not clear why DDFH2/2, but not DDFH2/2-R/A shows a phenotype. This needs more explanation.

3. The generation of the conditional knockout is messy and has not been represented or analysed in enough detail. In figure S4 it is shown that the line was generated by double crossover, but according to the text, interpretation of the PCR and the fact that revertants are seen in the population it is said to be a single crossover. Given this locus did not end up as expected a southern blot would

help with understanding what has happened at the MyoF locus.

4. In figure 8 presents data implicating MyoF on the positioning of the centrosomes and it is mentioned that one centrosome often ends up at the posterior end. This is hard to determine without labelling of the nucleus and marking the periphery of the parasite.

5. Fig 2D +CD - the green image is overexposed and should be replaced with a new one.

6. Fig 3 Is rhoptry positioning also reliant on ADF and Profilin? Do these two mutants also have a large residual body?

Minor Points:

1. The title is not wholy consistent with the findings. It is demonstrated and discussed that this actomyosin system is somehow important in centriole positioning. Would it not be appropriate to include this in the title?

2. The plastid is derived by secondary endosymbiosis. The use of 'double-endosymbiotic' is found in the first sentence of the abstract. This should be changed.

3. Citations throughout the manuscript are not well used. For example: 1. Fichera and Roos argued that the apicoplast was derived from a green algae, and not a red as used (P3). 2. There is also several self citations (eg Frenal et al) that are not appropriately used. 3. He et al 2001 EMBO and He 2001 JBC are the best citations for the delayed death phenotype but are not used. 4. Only self citations are used to reference the important role that the actomyosin motor system plays in parasite motility, no mention of work from the Sibley group here.

4. The study uses the processing of nuclear-encoded apicoplast proteins as a measure of the organelles biogenesis. It would be helpful if the authors gave introduction to this is the introduction section.

5. Dicussion: I'm not sure I understand why it is concluded that Golgi segregation is not affected upon perturbation of the actomyosin system?

Referee #3 (General Remarks):

This paper describes for the first time a role for TgMyo2F in the positioning of centrosomes and apicoplast inheritance in Toxoplasma gondii. Previous studies had shown that actin polymerization is required for parasite motility and cell invasion. Here, the authors show that actin polymerization is required for apicoplast inheritance in several Apicomplexans. In contrast, In contrast, using an inducibel KO approach confirmed by the expression of dominant-negative constructs of MyoF, they found that while being essential for apicoplast inheritance and parasite survival, MyoF is not required for motility, invasion, or egress from infected cells.

This is a potentially interesting study showing a role for MyoF in apicoplast inheritance. Unfortunately, I have mixed feelings about the clarity and impact of the messages that are delivered. The actin cytoskeleton has been involved in organelle recycling, so part of the data are not entirely unexpected. Also, although MyoF appears to be implicated in specific steps of apicoplasts inheritance, it is not clear how direct its involvement is. The work remains largely descriptive at this stage, and mechanical insights into how TgMyoF controls apicoplast inheritance would significantly improve the quality of this study.

1. How does MyoF accumulates along the side of the apicoplast?
2. Is the MyoF motor function involved in apicoplast inheritance?
3. How does MyoF maintains the centrosome in close proximity?
4. Molecular clues could be provided by looking at partner interacting with the MyoF WD40

domain.
5. Is the accumlulation of organelles in residual bodies dependent on the positioning of centrosomes?
6. The fact that only the overexpression of FRM2 FH2 domain interferes with apicoplast inheritance suggests that FRM2 play a function. If this inheritance of apicoplast was only due to an increase in the number of actin filaments, FRM1 FH2 overexpression would also impact on apicoplast inheritance, an aspect that could probably be investigated further in this study.
7. Figs 2, 3, 7 need quantification.
8. The authors show that actin polymerization and actin nucleators are involved in loss of apicoplasts. Then, they go on and investigate the role of MyoF. Yet, the rationale for looking at a conserved myosin motor and specifically MyoF is not clear. Actin dynamics may indirectly be organization of the cytoskeletal structures required for proper positioning of organelles independent of myosin motors. Did the authors looked Did the authors looked at the localization of other myosins at the apicoplast and into their role in apicoplast inheritance?
9. The effects of Cytochalasin D and jasplakinolide on the localization of MyoF should be analyzed.
10. How specific to MyoF is the inhibition mediated by the regulated expression of the DD-MyoF tail? Did the authors test the its association with TgMyoA?

Minor comments:

1. Fig 2A : the marker GAP45 is not defined.
2. Fig 2B: the accumulation of Cpn60 precursor has to be quantified
3. The introduction is a wordy and could probably by be shortened by 50 %.
4. In Fig4C MyoF is localized trhoughout the cytoplasm, so the conclusion that MyoF is localized at the vicinity of Apicoplast is not convincing. The function of MyoF localized at the extremity of dividing parasites should be commented.
5. Fig 5B: it seems that MyoF3Ty is more expressed than iMycMyoF, and that MyoF3Ty is more expressed at the periphery. Can the authors comment?
6. Do the different tags affect the MyoF function?
7. It is stated in the text p10 line 15 that "mitochondrial content.... Accumulated in the residual bodies", which is inconsistent with the legend to Fig5E
8. Fig 6B and S5A: why are the controls different (ie actin and catalase)?
9. Fig S5C: the meaning of "RH" has to be explained
10. It is not clear in the Fig 8D-E that the centrosomes are associated with the daughter cells. This has to be quantified.

Referee #4 (General Remarks):

In the current study the authors investigated the involvement of actin, actin binding factors and class XXII myosin F in apicoplast inheritance of two apicomplexans. The paper is well written and the experiments are well performed and conclusive in showing a role for myosin F in apicoplast duplication although the exact mechanism stays elusive.

The authors made use of fluorescence and electron microscopy and Western blots. Using these approaches, previously generated constructs were employed to assess the effect on apicoplast transmission. Such approaches were suitable for the research questions asked. These were appropriately performed and the inducible regulation of genes served as a good means to observe effects on division processing. Complementing the imaging component with Cpn60 processing analysis provided an additional means to verify the observations. The reviewer has no major criticism with the way in which the study was performed. However, the authors could consider presenting their Western blot results in a more quantitative bar graph format as this might make their results clearer.

Given the general nature of the EMBO Journal, I would recommend enhancing the introduction and discussion to be of interest to a wider audience by including some background of how the results on myosin F-apicoplast inheritance relates to other organisms and what is known about the roles of actin and myosin in mitochondrial and/or chloroplast inheritance.

Videos should be cropped to enlarge the cells of interest, currently there is a lot of black space.

It would be good to have additional data on "The accumulation of rhoptry and microneme contents in the residual bodies also suggests the involvement of TgMyoF in either anchoring or trafficking of these organelles."

Recent paper by Andenmatten on actin involvement in apicoplast inheritance should be cited

Minor text edits:

'loss the apicoplast (Figure 6D)' should be 'lost'

'a found within considerably enlarged residual bodies (Figure 7B)' should be 'are found...'

1st Revision - authors' response 19 April 2013

Responses to the reviewers

Referee #1

A role for an actomyosin motor and apicoplast inheritance is described. Actin inhibitor cytocholasin D is shown to perturb apicoplast inheritance in Toxoplasma gondii, Plasmodium falciparum, P. berghei. Knockdowns of profilin, actin depolymerization factor and a forming also perturbed apicoplast inheritance. A specific myosin (MyoF) is shown to accumulate near apicoplasts during division. Two knockdowns of MyoF were undertaken: a knock-in of a regulatable promoter, and expression of a dominant negative dysfunctional competitor MyoF. Both knockdowns exhibit perturbation of apicoplast inheritance. The experiments are well designed, clearly presented and the interpretations are valid. This work demonstrates an important new role for actomyosin in the accurate segregation of daughter organelles into daughter cells in the Phylum Apicomplexa.

Whether or not such a system operates in the plastids of algae and plants, or is restricted to Phylum Apicomplexa with its bizarre cytokinesis, is not discussed.

The molecular mechanism by which the apicoplast is inherited in Apicomplexa is not necessarily similar to the one governing the inheritance of chloroplasts occurring in plants or algae. Indeed the situation is considerably complicated by the presence of two additional membranes originating from the secondary endosymbiotic event (engulfement of a photosynthetic eukaryote). In contrast, it is plausible that the large groups of alveolates that possess red alga-derived plastids (Cryptomonads, Haptophytes, Stramenopiles Dinoflagellates and Apicomplexa) might share a common mechanism for their plastid inheritance. We have added some comments to address this point in the discussion.

I have made various corrections, suggestions and queries directly on a hard copy of the manuscript and a PDF has been sent to the editor and should be supplied to the authors.

Answers and corrections to the queries and comments indicated on the hard copy of the manuscript:

- All spelling mistakes have been corrected
- Page 3: The “clumsy sentence” has been rephrased
- Meissner Curr Biol 2009 was actually included in the list of references: it corresponds to Breinich et al. 2009.
- Page 5: The “unclear sentence” has been rephrased.
- Page 9: to the question “*TgMyoA is not implicated in apicoplast inheritance*.” Why?

  We were able to exclude any impact of MyoA depletion on apicoplast inheritance. The data supporting this claim are presented in the new Figure S3A-B**.**

- Page 12: The sentence “lacking sense” has been rephrased.

- Figure 7A absence of visible residual bodies: We have re-organized the Figure 7 with 4 panels 7A-C to avoid any discrepancy with the figure legend.
- Page 13: A quantitative analysis (in %) of parasite in the up/down position is indicated.
- References for inhibition of apicoplast protein synthesis by tetracycline and doxycycline are added to the text. The antibiotic effect of ATc impacts on parasite survival only at a very high concentration (Meissner et al, 2001). To our knowledge, this study is the first report showing the effect of high doses of ATc on apicoplast biogenesis in *Toxoplasma gondii.*
- Legend of Figure 4 corrected as requested.

I also suggest a number of changes to clarify things for the reader

Mitochondria are shown in some parts of Fig 1 but not all. As it stands, one might infer that mitochondria disappear and are generated de novo after endodyogeny is completed, which is not the case. Fix this.

We agree that this can be a source of confusion. In order to simply the scheme and to avoid ambiguity we have removed the mitochondrion from the scheme. For the same reason, the ER and the subpellicular microtubules are also not represented.

Throughout. The formal name of this group of parasites is Phylum Apicomplexa. The authors use the name 'Apicomplexan' in places. Strictly speaking, apicomplexan is an adjective (i.e. apicomplexan parasite) not a name and shouldn't be capitalized.

We have corrected throughout the text accordingly.

Fig2B (and elsewhere). Other users of the Cpn60 processing assay to analyze apicoplast defects have identified the lower mass band as mitochondrial Cpn60 (Agrawal JBC 2009). Do the authors disagree with this interpretation? Why?

Agrawal identified the lower mass band to "likely represent mitochondrial Cpn60". The exact identity of this band was never proved formally. We could not observe a mitochondrial staining using this antibody. Due to the lack of evidence we prudently did not assess a specific protein to this band.

Fig 2E. The minus cytocholasin D parasite(s) look(s) to have already egressed, but the lack of a differential interference contrast image makes it hard to know. In the Fig 2E panel where the effect of the addition of cytocholasin D is shown a bright field image is supplied for this schizont. Be consistent. Show bright field images for both and preferably the same stage.

The experiment with *Plasmodium berghei* was repeated and new images with higher resolution are now presented in the new Figure 2E**.** Parasites are in the same stage and bright field images are supplied.

Page 9. What system of nomenclature is being used for genes versus proteins? I deduce that the gene name is in italics and the protein in non-italic font. Even so, the sentences sometimes appear to be referring to a gene when they are talking about a protein and vice versa.

We have carefully revised the manuscript, figures and figure legends to adhere uniformly to the as proposed genetic nomenclature in *Toxoplasma gondii* according to Sibley et al Parasitol Today 1991 (in italic for the name of the gene and non-italic for the name of the protein).

Throughout. The 'w' in western blot should only be capitalized when used at the beginning of a sentence. Although convention allows capitalization, it is preferable to perpetuate the commemoration of Edwin Southern by restricting capitalization to the proper noun - Southern blotting.

We have corrected "western" throughout the manuscript.

Figure 5 - The line pointing to pCpn60 is too high. It doesn't point to the level of the band.

Thanks for spotting this mistake. Upon the request by the reviewers to provide a more quantitative analysis of the two forms of Cpn60, we have generated biological triplicates and quantified the bands. The western blot in new Figure 5 has been replaced by a representative one of better quality.

Figure 7 - It is confusing to have four images and two figure labels (7A & 7B). Change to 7A-C. This occurs in many places in the figures. I appreciate that this is to keep subgroups simple within a compound figure, but it can be ambiguous. I would recommend that every image has a number/letter designation, but that's up to the editor.

We agree with the referee and we have now organized the new Figure 7 with 4 panels 7A-C. To our view the other figures did not require to be reorganized.

Page 14 - Why do the authors refer to 'mitochondrial content'? I appreciate that the marker (HSP70) is for a soluble matrix protein, but other markers used are similar and the location of the entire organelle is inferred from these markers.

T. gondii possesses a single tubular mitochondrion. Since the organelle is still intact and present in the mutant or drug treated parasites, we felt that it would be inappropriate and misleading to state that the organelle was found in the residual body and hence we referred to “mitochondrial content” instead. We have rephrased it and refer now to mitochondrial fragment present in residual bodies, supported by the data presented in Figure 7C.

I'm not a big fan of the 'horizontal' and 'vertical' terminology (page 13) for the orientation of the daughter cells during endodyogeny. I read this many times, but it just isn't intuitive. Would a clock face analogy work better? For instance, 12 o'clock for wild type, 6 o'clock, and occasional 9/3 o'clock for DDMyoF expressing parasites.

We have been struggling to find an adequate way to describe this phenotype. We have now opted for a simplified terminology and only kept the orientation up, up/down and down.

Fig 9B & 9C - Again the mitochondria disappear and magically reappear after endodyogeny. Fix this.

Corrected, see comments above.

The Discussion needs to incorporate the connector between the apicoplast and centrosome (Francia PLoS Biol 2012).

Reference to this study was inadvertently omitted and has now been cited and discussed.

Referee #2

This paper by Jacot et al presents evidence for the role of the actomyosin cytosekeleton in the inheritance of the apicoplast - a relict non-photosynthetic plastid in the phylum Apicomplexa. In particular the authors present a role for a conserved class XXII myosin - MyoF in controlling apicoplast segregation. The authors primarily use Toxoplasma gondii to study this phenomenon but also show the applicability of their findings, where possible to the malaria-causing parasite Plasmodium spp.

This study will have a wide readership from scientists interested in either apicomplexan or myosin biology and therefore is appropriate for a EMBO Journal.

This study is well executed and is well controlled and their conclusions are largely warranted by the data presented. However, I believe a few issues should be addressed before its publication.

Major points:

1. The authors have focused the role of MyoF and actin dynamics in apicoplast segregation yet it is clear that there is a role for this system in the positioning of the rhoptries. This is mostly glossed over although presented and mentioned as work submitted in another paper. Is there any strong justification for leaving this work in? I think it detracts from the central message of the paper.

The implication of TgMyoF in rhoptry organelles positioning is reported in a separate article (Mueller et al, Cell Host & Microbe, 2013). This study describes the key role of TgARO in positioning the rhoptries to the apical pole of the parasites and TgMyoF has been identified as an interacting partner of TgARO. There is no direct overlap with the work presented here and in consequence the direct implication of TgMyoF on the apical positioning and anchoring of rhoptries is not described elsewhere.

We feel that it would be logical and appropriate to describe here comprehensively the phenotype of TgMyoF-iKO and DDMyoF-tail stabilization and thus to include the observed impact on rhoptries. We have reduced the description of this phenotype to its minimum to avoid distraction from the other impact of MyoF on the centrosome positioning and apicoplast inheritance.

2. Figure S2 (discussed on p8): It is not clear to me why this section is not presented in the main text. It is also not clear why DDFH2/2, but not DDFH2/2-R/A shows a phenotype. This needs more explanation.

The data on DDFH2/2 are now presented in the main figure. The results obtained with DDFH2/1 and DDFH2/2-R/A used as controls are kept in Figure S2.

To our view the analysis of the formin FH2 dominant mutants is interesting and intriguing since loss of apicoplast is occurring exclusively in presence of an excess of the FH2 domain of TgFRM2 but not with the presence of the FH2 from TgFRM1.

Expression of DDFH2/2 has two distinct impacts on the parasites i) FH2/2 can form heterodimers with TgFRM2 and poison specifically the function of this formin (Daher et al, 2010) ii) FH2/2 can also form homodimers and act as an uncontrolled nucleator of actin polymerization. DDFH2/2-R/A is mutated in the actin binding which results in a reduction of at least 90% of its nucleating activity (Daher et al, 2010). In consequence DDFH2/2-R/A only blocks TgFRM2 function but does not lead to deregulated actin polymerization. DDFH2/2-R/A is included here as control, which supports the view that controlled actin dynamics is important for apicoplast inheritance.

3. The generation of the conditional knockout is messy and has not been represented or analyzed in enough detail. In figure S4 it is shown that the line was generated by double crossover, but according to the text, interpretation of the PCR and the fact that revertants are seen in the population it is said to be a single crossover. Given this locus did not end up as expected a southern blot would help with understanding what has happened at the MyoF locus.

We have modified Figure S4 and represented schematically the modified *MyoF* locus in the new Figure S4C.

The inducible knockdown mutant has recombined in the locus of *TgMyoF* but led likely to the insertion of more than one copy. The attempt to sort out the 5' recombination event by southern blot was not successful but revealed a band in accordance with such multiple integration.

We have also assessed the down regulation of the inducible copy by quantitative RT-PCR. The data are included on the new Figure S5D. Furthermore, during the course of the revision we succeeded to isolate a clone where double recombination occurred (Figure 1, Response to the referees' comments). Regulation of iMycMyoF was also confirmed by western blot at the expected mass. This new MyoF-iKO was investigated and revealed the same phenotypes i.e. apicoplast loss and accumulation of organelles in residual bodies. Intracellular growth, invasion and daughter cell orientation assays were also repeated and confirmed the previous experiments.

4. In figure 8 presents data implicating MyoF on the positioning of the centrosomes and it is mentioned that one centrosome often ends up at the posterior end. This is hard to determine without labeling of the nucleus and marking the periphery of the parasite.

We have repeated those experiments and documented by including DAPI staining. We also transiently transfected the parasites with the plasmid IMC-tomato but the quality of the pictures was lower than the ones with ISP1 antibodies. The results are presented in the new Figure 8.

5. Fig 2D +CD - the green image is overexposed and should be replaced with a new one.

The experiments were repeated with *Plasmodium falciparum* and documented again. We have replaced the image in the new Figure 2D and hope it is found to be of better quality.

6. Fig 3 Is rhoptry positioning also reliant on ADF and Profilin? Do these two mutants also have a large residual body?

Rhoptries were only slightly affected in parasites depleted in ADF or PRF. Accumulation of rhoptries in residual bodies was only observed in a low percentage of parasites. The same modest impact on rhoptries was seen after stabilization of DDFH2/2. The significantly enlarged residual bodies were only observed when interfering with MyoF function either by depletion of the motor (MyoF-iKO mutant) or following stabilization of the dominant mutant DDMyoF-tail. Data are presented in Figures S2D.

Minor Points:

1. The title is not wholy consistent with the findings. It is demonstrated and discussed that this actomyosin system is somehow important in centriole positioning. Would it not be appropriate to include this in the title?

We have revised the title according to this reviewer suggestion.

2. The plastid is derived by secondary endosymbiosis. The use of 'double-endosymbiotic' is found in the first sentence of the abstract. This should be changed.

We have rephrased the sentence in the abstract and replaced 'double-endosymbiotic' by secondary endosymbiosis.

3. Citations throughout the manuscript are not well used. For example: 1. Fichera and Roos argued that the apicoplast was derived from a green algae, and not a red as used (P3).

We have rephrased the paragraph to separate the references related to the discovery of the organelle form those establishing its red alga origin.

2. There are also several self-citations (e.g. Frenal et al) that are not appropriately used.

Frenal et al 2010 has indeed been used and abused in this manuscript. We have removed this citation where it was inappropriate such as at the bottom of page 5 where we now only cite the original articles that demonstrated the implication of TgMyoA in gliding and in page 11 where it has been replaced by Polonais et al Traffic 2011.

3. He et al 2001 EMBO and He 2001 JBC are the best citations for the delayed death phenotype but are not used.

He et al., 2001 EMBO was already accurately cited on page 4, as one of the studies reporting on the phenomenon of "delayed death" phenotype.

He et al., 2001 JBC "*Targeting and Processing of Nuclear-encoded Apicoplast Proteins in Plastid Segregation Mutants of Toxoplasma gondii*". In this paper the authors investigated apicoplast

protein synthesis, targeting, and processing in the presence or absence of the organelle. To our view this does not refer to an original study describing the delayed death phenotype. This paper was cited latter in the paragraph introducing the targeting and processing of nuclear-encoded apicoplast proteins.

4. Only self citations are used to reference the important role that the actomyosin motor system plays in parasite motility, no mention of work from the Sibley group here.

The Sibley's laboratory has made seminal contributions establishing the role of parasite actin in motility and invasion. They also made the initial observation of the implication of myosin in gliding motility based on inhibitors and heterologous antibodies but did not identified the motor implicated. We have introduced citations acknowledging the key contributions. Mehta S, and Sibley LD. Mol Biol Cell. 2011 reports the implication of TgADF and was already cited for the mutant strain used in this study. We have added Wetzel DM et al, Mol Biol Cell. 2003 *Actin filament polymerization regulates gliding motility by apicomplexan parasite*. Dobrowolski JM et al Mol Microbiol. 1997 *Participation of myosin in gliding motility and host cell invasion by Toxoplasma gondii.* Dobrowolski JMand Sibley LD. Cell. 1996 *Toxoplasma invasion of mammalian cells is powered by the actin cytoskeleton of the parasite.*

4. The study uses the processing of nuclear-encoded apicoplast proteins as a measure of the organelles biogenesis. It would be helpful if the authors gave introduction to this is the introduction section.

We have added a paragraph to introduce this notion in the introduction section.

5. Discussion: I'm not sure I understand why it is concluded that Golgi segregation is not affected upon perturbation of the actomyosin system?

Based on the various strategies used in this study, actin dynamics perturbation by drugs, depletion of MyoF and stabilization of DDMyoF-tail, we never observed a defect in Golgi inheritance based on detection of GRASP-YFP. The Golgi is always found in close proximity of the centrosomes regardless their orientation during division and both organelles are encapsulated in the forming daughter cells. Parasites lacking the apicoplast still possessed a Golgi.

Referee #3

This paper describes for the first time a role for TgMyo2F in the positioning of centrosomes and apicoplast inheritance in Toxoplasma gondii. Previous studies had shown that actin polymerization is required for parasite motility and cell invasion. Here, the authors show that actin polymerization is required for apicoplast inheritance in several Apicomplexans. In contrast, In contrast, using an inducible KO approach confirmed by the expression of dominant-negative constructs of MyoF, they found that while being essential for apicoplast inheritance and parasite survival, MyoF is not required for motility, invasion, or egress from infected cells.

This is a potentially interesting study showing a role for MyoF in apicoplast inheritance. Unfortunately, I have mixed feelings about the clarity and impact of the messages that are delivered. The actin cytoskeleton has been involved in organelle recycling, so part of the data are not entirely unexpected. Also, although MyoF appears to be implicated in specific steps of apicoplasts inheritance, it is not clear how direct its involvement is. The work remains largely descriptive at this stage, and mechanical insights into how TgMyoF controls apicoplast inheritance would significantly improve the quality of this study.

1. How does MyoF accumulates along the side of the apicoplast?

2. Is the MyoF motor function involved in apicoplast inheritance?

3. How does MyoF maintains the centrosome in close proximity?

Obviously these are key, central but not trivial questions around the mode of action of MyoF. They are pertinent and require considerable investigations. Questions 1 and 3 directly relate to the identification of TgMyoF interacting partners. We have intensively searched form them without success so far (see point 4 below).

With regard to question 2, we feel that the implication of MyoF in apicoplast inheritance is what our study establishes. The questions is harder to address if the reviewer is asking specifically about the ATPase activity of MyoF in contrast to a more structural contribution (tension) in organelle inheritance. The only unambiguous way to address the point would be to generate a smart mutant that would kill the ATPase activity. In absence of *in vitro* activity assay it would be dangerous to predict the effect of point mutations based on what is known about the myosins class I, II, V and VI. Importantly, the fact that overexpression of DD-MyoF-tail is leading to a severe defect recapitulating the conditional depletion of the motor speaks for the critical need for a functionally active homodimer. The use of the myosin APTase inhibitor BDM has been inconclusive because i) there is no absolute guarantee that it targets TgMyoF and its use at mM range is very deleterious for both the host cells and parasite survival.

4. Molecular clues could be provided by looking at partner interacting with the MyoF WD40 domain.

We did perform a series of co-immunoprecipitations (co-IPs) aiming at identifying MyoF interacting partners, using DDMyoF-WD40 as bait. We tested several conditions with either intracellular or extracellular parasites and with various co-IP buffers. Several bands were sent for mass spectrometry however it led to the identification of apparently unrelated proteins so far. The interaction of MyoF with the potential partners might be only transient during division and might therefore be difficult to catch. We also attempted co-IPs with MyoF-3Ty and TgMyoF-iKO but the numerous degradation products complicated the identification of potential partners. We are currently exploring alternative approaches including the use of cross-linkers and also bacterial recombinant WD40 domains.

Coming from a different angle, we have established that Armadillo Repeats-Only protein (TgARO) binds to TgMyoF (Mueller et al, 2013). While this interaction has been reproducibly validated using different versions of TgARO, we failed to co-IP TgARO by pulling on TgMyoF, illustrating the technical challenge of precipitating this large motor (216 kDa) which is likely interacts with multiple partners at different time point during the cell cycle.

5. Is the accumulation of organelles in residual bodies dependent on the positioning of centrosomes?

We suspect that this is the case. All experimental approaches that affect MyoF function impact on centrosomes positioning, compromise the polarity of the cell division and lead to accumulation of organelles in residual bodies. In contrast, the depletion of TgARO, which affects the positioning of the rhoptry organelles (likely implicating MyoF function) leads to a dispersion of the organelles in cytosol but not the formation of enlarged residual bodies.

6. The fact that only the overexpression of FRM2 FH2 domain interferes with apicoplast inheritance suggests that FRM2 play a function. If this inheritance of apicoplast was only due to an increase in the number of actin filaments, FRM1 FH2 overexpression would also impact on apicoplast inheritance, an aspect that could probably be investigated further in this study.

Yes indeed this is a puzzling and rather unexpected observation.

The selective effect of FH2/2 suggests that TgFRM2 but not TgFRM1 is implicated in this aspect of actin function. In contrast, expression of DDFH2/2-R/A (mutated in the actin binding) does not affect the apicoplast inheritance although it impairs FRM2 function via the formation of an inactive heterodimer (Daher et al, 2010). It is plausible that overexpression of FH2/2-R/A is not sufficient to block all the endogenous FRM2 molecules but it is premature to conclude. In our view only the complete deletion of TgFRM2 could clarify this question.

Alternatively, the FH2 domain of TgFRM2 (FH2/2) was recently demonstrated *in vitro* to be 10 times more potent nucleator of *Toxoplasma* actin than TgFRM1 (FH2/1) (Skillman et al, 2012) which could explain the differential effects observed on the apicoplast *in vivo*.

7. Figs 2, 3, 7 need quantification.

Western blots showing the accumulation of the precursor form of Cpn60 with the concomitant decrease of the mature form were quantified in the Figures 2 and 3. The values obtained from biological triplicates are included in the main figures.

Figure 7 shows EM data revealing the presence of intact organelles in the residual bodies. We are unclear, which quantification would be required here. The presence of enlarged residual bodies is visible by IFAs and a large number of vacuoles (>50%) exhibit this striking phenotype.

8. The authors show that actin polymerization and actin nucleators are involved in loss of apicoplasts. Then, they go on and investigate the role of MyoF. Yet, the rationale for looking at a conserved myosin motor and specifically MyoF is not clear. Actin dynamics may indirectly be organization of the cytoskeletal structures required for proper positioning of organelles independent of myosin motors. Did the authors look at the localization of other myosins at the apicoplast and into their role in apicoplast inheritance?

First of all, we reasoned that a directed (non-random) organelle movement by actin rocketing would unlikely lead the inheritance of a large, single organelle. The absence of visible actin filaments in Apicomplexa made this option even more unlikely. The fact that segregation of the apicoplast in the human and rodent malaria parasites was sensitive to drugs affecting actin polymerization led us to postulate that a shared myosin could be implicated. Beside MyoA, MyoF is the only motor conserved across the phylum.

T. gondii possesses eleven myosins and the deletion of *MyoA, MyoB/C* (unpublished) and *MyoD* do not alter apicoplast inheritance. We have indeed examined the localization of the remaining motors and only MyoF exhibited a subcellular distribution compatible with apicoplast inheritance.

9. The effects of Cytochalasin D and jasplakinolide on the localization of MyoF should be analyzed.

The impacts of drugs perturbing actin polymerization have been investigated on parasites expressing the endogenously tagged MyoF (MyoF-3Ty). New data are included in the Figure S3D-E.

10. How specific to MyoF is the inhibition mediated by the regulated expression of the DD-MyoF tail? Did the authors test the its association with TgMyoA?

The envisioned mode of action to generate a dominant effect with a myosin tail are i) titration of the myosin light chains (MLCs) via the neck domain ii) sequestration of the cargo binding to the tail domain iii) the formation of an inactive heterodimer via the coiled-coil domain.

i) The construct used to assess TgMyoF function (DD-MyoF-tail) lacks the neck domain containing the IQ motifs known to bind to MLCs. This eliminates a major risk of targeting other myosin motors by titrating out shared MLCs.

ii) MyoF and MyoA are not anticipated to share the same cargo since MyoA actually is devoid of tail and use the neck and MLC1/GAP45 to localize to the pellicle. Moreover depletion in TgMyoA has no impact on apicoplast inheritance (Figure S3)

iii) Among the eleven myosins present in *T. gondii* only MyoG and MyoF contain obvious coiled-coil domains susceptible to form a dimer. Overexpression of a DDMyoG-tail using the same strategy as for DDMyoF-tail did not reveal any phenotype in apicoplast inheritance (unpublished). Finally, MyoA is a single headed motor.

Minor comments:

1. Fig 2A : the marker GAP45 is not defined.

The Gliding-Associated Protein 45 (GAP45) is now defined.

2. Fig 2B: the accumulation of Cpn60 precursor has to be quantified.

Biological triplicates of the western blots have been quantified and the values are added to the figures (below the western blots).

3. The introduction is a wordy and could probably by be shortened by 50 %.

We have tried to produce a more concise introduction while adding also some specific information that was requested by the reviewers.

4. In Fig4C MyoF is localized throughout the cytoplasm, so the conclusion that MyoF is localized at the vicinity of Apicoplast is not convincing. The function of MyoF localized at the extremity of dividing parasites should be commented.

In dividing parasites we can see a clear concentration of MyoF around the apicoplast and at the vicinity of nascent daughter cells in addition to its spread and peripheral localization.

5. Fig 5B: it seems that MyoF3Ty is more expressed than iMycMyoF, and that MyoF3Ty is more expressed at the periphery. Can the authors comment?

Obviously, immunofluorescence stainings were obtained from different antibodies detecting different epitope tags (Myc/Ty), positioned in different numbers (1/3) on distinct locations (Nterm/Cterm) on the large protein encoded by genes controlled by different promoters (TetO/MyoF) and hence can hardly be compared!

We certainly cannot conclude anything about the level of expression of iMyc-MyoF versus MyoF-3Ty. With regard to localization of these tagged proteins, we observed a substantial cell cycle dependent variation of the pattern, which complicates the comparison. Despite this limitation, we consistently see MyoF at the parasite periphery is non-dividing parasites with both constructions. We have attempted to generate antibodies against TgMyoF without success.

6. Do the different tags affect the MyoF function?

To undertake functional dissection of a gene as conducted here via conditional depletion and overexpression of dominant mutants, it is imperative to be able to detect selectively the transgenes, Introduction of a short epitope tag is one of the less invasive approach however one can not rule out some impact on localization and or function.

In the case of TgMyoF, the introduction of a C-terminal epitope tag in the endogenous locus showed not detectable phenotype, suggesting the tag does not perturb the function of the motor in any significant way. Epitope tagging at the N-terminus of TgMyoF was achieved for the generation of a conditional knockout. The transgenic parasites expressing the inducible copy appear to be slightly less fit compared to wild type parasites (although they do not lose the apicoplast in absence of ATc). However, we cannot discriminate a possible impact of the timing and level of expression of the inducible copy versus the presence of a tag at the N-terminus.

In summary we cannot formally exclude that the tags affect MyoF function however if there is a perturbation it would be very modest and would not impact on the conclusions.

7. It is stated in the text p10 line 15 that "mitochondrial content.... Accumulated in the residual bodies", which is inconsistent with the legend to Fig5E

This discrepancy between the figure and the figure legend has been fixed with the legend of Figure 5 rephrased.

8. Fig 6B and S5A: why are the controls different (i.e. actin and catalase)?

DDMyoF-WD40 migrates around 60kDa and catalase (used as loading control) migrates also around 60kDa. Since the loading controls are detected on the same membrane we opted for actin as loading control, which migrates faster, to avoid interference between the two signals. To our view both catalase and actin work equally well as loading controls and importantly the antibodies are sensitive and recognize exclusively the corresponding protein of parasite origin.

9. Fig S5C: the meaning of "RH" has to be explained.

"Sabin (1941) reported toxoplasmosis in a 6-y-old boy from Cincinnati, OH. *An asymptomatic child with initials of R.H. was hit with a baseball bat on October 22, 1937. He developed a headache 2 days later and convulsions the day after. He was admitted to the hospital on the seventh day but without obvious clinical signs. Except for lymphadenopathy and enlarged spleen, nothing abnormal was found. He then developed neurological signs and died on the 30th day of illness. The brain and spinal cord were removed for histopathological examination and bioassay. Because of the suspicion of polio virus infection a homogenate of cerebral cortex was inoculated into mice. Toxoplasma gondii was isolated from the inoculated mice and this isolate was given the initials of the child and became the famous RH strain*." (Dubey, 2008). We have included the reference for the strain RH HXGPRT-KO in the materials and methods.

10. It is not clear in the Fig 8D-E that the centrosomes are associated with the daughter cells. This has to be quantified.

The best way to detect the centrosomes is achieved using centrin antibodies. Unfortunately the only anti-centrin antibodies used in some studies have not been made available to us (loss of the hybridoma). Alternatively the expression of a Centrin-RFP fusion gives a slightly more complicated picture. That being said centrosomes have always been found in association with the daughter cells. A recent study has demonstrated that daughter cell formation depends on the presence of a striated rootlet fiber that emerges from the centrosomes immediately after their duplication. The daughter cells form at the distal tip of the fiber, which associates with the conoid and the polar ring. Disruption of two components of this fiber (TgSFA2 and TgSFA3) blocks daughter cell formation without affecting mitosis (Francia et al, 2012). In Figures 8D-E parasites are clearly not impaired in division and the centrosomes remain associated with the forming daughter cell.

Referee #4

In the current study the authors investigated the involvement of actin, actin binding factors and class XXII myosin F in apicoplast inheritance of two apicomplexans. The paper is well written and the experiments are well performed and conclusive in showing a role for myosin F in apicoplast duplication although the exact mechanism stays elusive.

The authors made use of fluorescence and electron microscopy and Western blots. Using these approaches, previously generated constructs were employed to assess the effect on apicoplast transmission. Such approaches were suitable for the research questions asked. These were appropriately performed and the inducible regulation of genes served as a good means to observe effects on division processing. Complementing the imaging component with Cpn60 processing analysis provided an additional means to verify the observations. The reviewer has no major criticism with the way in which the study was performed.

However, the authors could consider presenting their Western blot results in a more quantitative bar graph format as this might make their results clearer.

Biological triplicates of the western blots were quantified for accumulation of the precursor form of Cpn60 and concomitant decrease of the mature form in case of CD treatment, PRF-iKO, ADF-iKO, DDFH2, MyoF-iKO and DDMyoF-tail. The values have been included in the main figures.

Given the general nature of the EMBO Journal, I would recommend enhancing the introduction and discussion to be of interest to a wider audience by including some background of how the results on myosin F-apicoplast inheritance relates to other organisms and what is known about the roles of actin and myosin in mitochondrial and/or chloroplast inheritance.

We have paid a special attention to this point and broaden the relevance of the work in the introduction and discussion. To do so we have recapitulated what is known about chloroplast inheritance and also pointed out the complex situation involving the acquisition of a secondary endosymbiont in the groups of chromoalveolates.

Videos should be cropped to enlarge the cells of interest, currently there is a lot of black space.

The videos have been cropped as requested.

It would be good to have additional data on "The accumulation of rhoptry and microneme contents in the residual bodies also suggests the involvement of TgMyoF in either anchoring or trafficking of these organelles."

Data reporting the potential implication of TgMyoF in rhoptry organelles trafficking are cited and discussed. We shall point out here that we have revised our interpretation of MyoF-iKO when comparing it with the contrasting phenotype of ARO-iKO. Importantly, the depletion in TgARO does not lead to the formation of enlarged residual bodies and the dispersed rhoptries remain in the cytosol. In consequence we suspect that the accumulation of organelles in the residual bodies might be mainly an indirect consequence of the impaired positioning of the centrosomes. Also any evidence for a direct involvement of TgMyoF in trafficking of micronemes is lacking.

Recent paper by Andenmatten on actin involvement in apicoplast inheritance should be cited.

This paper was not out at the time of submission but is now cited.

Minor text edits:

'loss the apicoplast (Figure 6D)' should be 'lost'

'a found within considerably enlarged residual bodies (Figure 7B)' should be 'are found...'

Corrections done.

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