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Structural organization of the C1b projection within the ciliary central apparatus

Kai Cai, Yanhe Zhao, Lei Zhao, Nhan Phan, Yuqing Hou, Xi Cheng, George Witman and Daniela Nicastro
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MS TITLE: Structural organization of the C1b projection within the ciliary central apparatus

AUTHORS: Kai Cai, Yanhe Zhao, Lei Zhao, Nhan Phan, George Witman, and Daniela Nicastro
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 favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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.

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Reviewer 1

Advance summary and potential significance to field

In this manuscript, Cai and colleagues from the Nicatro and Witman labs characterize the effects of mutations in two genes (*FAP42* and *FAP246*) on the biochemical composition and structure of flagella, and on flagellar motility, in the model organism *Chlamydomonas reinhardtii*. Both gene products were previously identified as subunits in the C1b complex associated with the flagellar central pair microtubules a major regulatory structure in motile cilia.

Overall, this is an interesting paper that advances our understanding of the ultrastructure of ciliary central pair complexes. Both proteomics data and cryoEM data are of the highest quality and carefully interpreted, and provide strong support for the summarizing model presented for localization of individual proteins within the large C1b complex. The content is highly appropriate for the Journal of Cell Science.

Comments for the author

The paper could be improved with attention to a few points. The primary factor that seems to be missing is a connection between this data and what has previously been reported, much of it by the Witman and Nicastro labs which would put these new results in context.
First, both mutations were shown to have modest effects on swimming velocity, similar to the effects of the *cpc1* mutation that was previously characterized and that completely disrupts the C1b complex. As the authors go on to show that these new mutations generate smaller structural defects than *cpc1*, one might expect that they would closely phenocopy *cpc1* or perhaps have a lesser phenotypic effect.

Therefore it was disappointing that no direct comparisons were made between these new strains and *cpc1*. For example, the slow swimming phenotype of cpc1 was previously shown to be mostly due to reduced beat frequency. Is the reduced swimming speed of these mutations due to changes in beat frequency or to changes in waveform? The swimming paths of the new mutant strains were described as less linear than those of wild type cells, a phenotype that was not previously associated with *cpc1*, however no direct comparisons were made between the motility phenotypes of these new mutants and *cpc1*. These caveates should be noted.

Second, in their proteomics work the authors note reductions in two proteins that were previously identified as part of the C1b complex, but that also occur elsewhere in cilia, HSP70 and enolase. Once again, without a direct comparison to *cpc1*, the authors cannot tell whether the reductions in the ratios of these proteins in *fap4*2 vs wild type axonemes (35% reduction for enolase, 60% for HSP70) represents a loss of the entire amount of each protein normally present in the C1b complex. If the *fap42* mutation completely disrupts the association of these two proteins with C1b then some of the missing structure in the cryoEM averages should correspond to these proteins. This mass is not trivial, as the total of the two proteins is 123 kDa, similar to that of FAP246 (120 kDa). At the least the authors should note their inability to rule out the possibility that some of the missing structure in their *fap42* tomograms could represent these proteins. Perhaps this represents the difference between the estimated total of 550 kDa and the observed total of 450 kDa for the pillar (p. 12 last paragraph)? In addition, could the reduction in swimming speed could be due mostly to reductions in ATP concentrations in the ciliary compartment, from loss of enolase activity, as suggested by Mitchell et al., 2005? These possibilities should be discussed in the interpretation of the presented data.

Third, because *Chlamydomonas* is a model organism and has provided groundbreaking advances in ciliary ultrastructure, the results here are important whether the proteins under analysis are broadly distributed phylogenetically or not. However, it would provide the reader with a greater ability to follow the relative importance of these results if some of the information on phylogeny, previously so carefully analyzed by the Witman lab, and on the relative conservation of structure, previously analyzed by the Nicastro lab, were referenced here. In particular, the bracket structure is completely missing in sea urchin (Carbajal-Gonzalez et al., 2013), which is not surprising as the FAP246 and FAP413 proteins assigned to this structure here are limited to bikonts and green algae respectively, and so are completely missing in metazoa (Zhao et al., 2020).

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More surprising and also worthy of comment, the beam structure is here identified with the FAP42 protein. Although similar structures occur in sea urchin (Carbajal-Gonzalez et al), FAP42-related genes have a quite limited distribution among Cryptophyte and Haptophyte lineages and do not appear in metazoa (Zhao et al). The discussion should be expanded to include some information on the applicability of these data to other organisms.

Minor points:
1. Table 1 indicates proteins that are "significantly" reduced in bold print, but no criteria for significance were described.

2. p. 10, in describing the results presented in Fig. 2S, class3, "base" should be "tip".

3. Discussion, p. 14, in discussing the effects of their mutations on remaining C1b and C1f structures, the authors say "All these structures have a 16 nm periodicity…". However, C1f clearly has a 32 nm periodicity with two different structural shapes alternating along the C1 microtubule (as seen, for example, in Fig. 2 D and H, Fig. 3 E and J and Fig. 4 D and H and Fig. S2 B, D and F).

4. Discussion, p. 18, the authors state that the C2b projection in fap42 did not show obvious positional instability or reduction, but C2b clearly appears to have lost density in *fap42* as seen in Fig. S2 E.

5. The extent of the dark blue coloration of the C1b pillar in isosurface images is inconsistent, extending to the C1 microtubule surface in the main text figures, but not in Fig. S2 or in movies S1 or S4.

6. In the legend to Fig. S2, fap42 should be set in Italics.

Reviewer 2

Advance summary and potential significance to field

Central pair apparatus plays important regulatory roles in ciliary beat. Mutations of central pair proteins are linked to important human diseases. The central pair structure is huge, extremely complicated and among one of the most challenging targets for modern microscopy. Lacking a detailed architecture of central pair has largely limited the current understanding of its regulatory roles. Any progress that can provide a clearer picture of this gigantic machinery will improve our understanding of ciliary beating. Cai et al. reported their new results on the architecture of a sub-complex localized in C1b. The methods, analysis and manuscript are of good quality in general.

Comments for the author

Major issues:
(1) A most critical issue is that the authors lack a strong proof for the currently proposed localization of FAP413. The only evidence that helped the author to reach the conclusion is the mass spectrometry results (Table 1), which show that FAP413 is almost lost in the fap42 or fap246 mutants. It does not necessarily support that FAP413 must interact FAP42 or FAP246 to form the triple-complex that the author referred to as FAP246-FAP413-FAP42. Unlike fap42 or fap246, of which the authors did a very good job on the structural characterization, the fap413 mutant was not even tested. It is normal that lacking a certain subunit in an intricate protein complex will affect many of its neighbors. Not only its neighbors, sometimes other proteins that seem to be far away can also be remotely affected. I therefore suspect that there is a possibility that fap413 is localized in a different region unless the authors provide a much stronger evidence. The concern applies to other proteins that the authors had discussed but just not stated as their conclusions in this manuscript.

Other comments:
(2) In the current, as well as previous studies, the authors estimated the mass of several local regions of the central pair complex. I appreciate the authors' efforts, but one thing that has not been well discussed is the stoichiometry of each protein. This can sometimes affect the

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localization of the proteins. I am not doubting the current model, but rather suggest the authors at least provide their own data on this issue or previous evidence or some kind of analysis on different possibilities to propose a most likely model.

(3) As a research article, the most important result of this manuscript is no doubt the localization and possible architecture of the protein complexes themselves. All the rest of discussions, possible interactions, regulatory mechanisms and sensory roles etc. etc. are entirely based on the reliability of the structure itself. Yet, the authors discussed many things based on their limited model, far beyond what the data can support. This is risky and may affect the manuscript if the basis itself has a problem. So, my suggestion is that the authors substantially weaken some of the discussions but solidify their structural basis first. It is indeed the result itself that matters in the long term. To discuss so much without a solid basis makes me a bit uncomfortable about the manuscript. For example, the author claim that the C1b may be involved in nucleotide cycles. This hypothesis itself makes sense and I do not necessarily disagree with the authors. The issue is why it is related to this manuscript if the authors did not provide any of their own evidence. Without the cryo-ET structure, we can still make hypothesis on possible roles of FAP42 and FAP246 without determining the cryo-ET structure? The author stated that 'the FAP246-FAP413-FAP42 subcomplex is part of a large interconnected CA network" and "the FAP246-FAP413-FAP42 subcomplex provides mechanical support". Again, the issue is: does the FAP246-FAP413-FAP42 ever exist? If not, why the authors discuss about all these possible roles.

(4) I suggest the authors slightly soften some of their statements and compact some of the unrelated details, but focus a bit more on the most important point and provide sufficient evidence. For example, in the section "FAP246 is localized to the C1b projection and forms a complex with FAP413". I was trying to find any possible evidence that FAP246 and FAP413 form a complex, but unfortunately failed to understand how they reached this conclusion. Other details are good, but not essential for the main conclusion of their sub-title.

(5) I have a minor suggestion on the movie S2 and S3. Could it be possible to obtain more classes and make smoother movies?

First revision

Author response to reviewers' comments

Please find below our point-by-point answers to the reviewers' comments & suggestion:
The editor and reviewers' comments are copied below (*italic black*/*gray*) and our point-by-point answers are in blue font (text cited from the manuscript is in *red italic font*). Where appropriate we refer to specific sections in the revised manuscript, where changes have been made.

From the editor:
MS ID#: JOCES/2020/254227
MS TITLE: Structural organization of the C1b projection within the ciliary central apparatus
AUTHORS: Kai Cai, Yanhe Zhao, Lei Zhao, Nhan Phan, George Witman, and Daniela Nicastro
ARTICLE TYPE: Research Article

Dear Dr. Nicastro,

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 favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

We thank the editors for this assessment and for their patience, granting us more time to finish the

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revisions. We performed a considerable amount of additional experiments, especially cryo-electron tomography of an additional C1b mutant (*fap413*) as suggested by one of the reviewers. In addition to the major changes highlighted in the resubmitted manuscript (red font), we have made minor changes (not highlighted) to the main text and figure legends to improve syntax. I hope you will find that we have addressed the reviewers' comments thoroughly and that the clarity of the manuscript is increased. We have also shortened the text to under 8000 words as requested.

Reviewer #1: *Advance Summary and Potential Significance to Field*:

In this manuscript, Cai and colleagues from the Nicatro and Witman labs characterize the effects of mutations in two genes (FAP42 and FAP246) on the biochemical composition and structure of flagella, and on flagellar motility, in the model organism Chlamydomonas reinhardtii. Both gene products were previously identified as subunits in the C1b complex associated with the flagellar central pair microtubules, a major regulatory structure in motile cilia.

Overall, this is an interesting paper that advances our understanding of the ultrastructure of ciliary central pair complexes. Both proteomics data and cryoEM data are of the highest quality and carefully interpreted, and provide strong support for the summarizing model presented for localization of individual proteins within the large C1b complex. The content is highl appropriate for the Journal of Cell Science.

We thank the reviewer for this positive assessment.

Reviewer 1 Comments for the Author:
The paper could be improved with attention to a few points. The primary factor that ***seems to be*** *missing is a connection between this data and what has previously been reported, much of it by the Witman and Nicastro labs, which would put these new results in context.*

First, both mutations were shown to have modest effects on swimming velocity, similar to the effects of the cpc1 mutation that was previously characterized and that completely disrupts the C1b complex. As the authors go on to show that these new mutations generate smaller structural defects than cpc1, one might expect that they would closely phenocopy cpc1 or perhaps have a lesser phenotypic effect. Therefore it was disappointing that no direct comparisons were made between these new strains and cpc1.

We thank the reviewer for this suggestion. We have now carried out a direct comparison of the swimming phenotypes between the *cpc1* and *fap246* mutant cells; the data are presented in the new supplementary figure S2D and described in the revised text on page 7. Briefly, as the reviewer predicted, the *fap246-1* phenotype closely phenocopies that of *cpc1-2,* but is less severe. Specifically, the beat frequency of *fap246-1* is reduced to 0.72 of that of WT, whereas beat frequency of *cpc1-2* is reduced to 0.56* of that of WT (Fig. S2). Our results are consistent with the smaller structural defect in *fap246-1* as compared to *cpc1*, which was shown to lack the entire C1b projection (Zhang and Mitchell, 2004).
[* please note that the beat frequency reduction that we observed in *cpc1-2* is slightly greater than that reported by Mitchell and Sale (0.72) (Mitchell and Sale, 1999) or Zhang and Mitchell (0.64) (Zhang and Mitchell, 2004), and may represent slight differences in experimental conditions or the use of a different *cpc1* allele].

For example, the slow swimming phenotype of cpc1 was previously shown to be mostly due to reduced beat frequency. Is the reduced swimming speed of these mutations due to changes in beat frequency or to changes in waveform?

As reported in the original version of our manuscript, swimming speed of *fap246-1* is reduced to 0.72 of WT. Our new data show that beat frequency of *fap246* is reduced to 0.76 of WT. Thus, we can deduce that the reduced swimming speed of *fap246* is due mainly to the reduction in beat frequency, as also was reported to be the case for *cpc1*. We describe the new data in the revised text on page 7.

The swimming paths of the new mutant strains were described as less linear than those of wild type cells, a phenotype that was not previously associated with cpc1, however no direct

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comparisons were made between the motility phenotypes of these new mutants and cpc1. These caveats should be noted.

To our knowledge, swimming paths of *cpc1* cells have not been reported previously. When we recorded the swimming paths of *cpc1-2* and *fap246-1* using high-speed video microscopy (400 frames/s), we found that cells of both mutants turned more frequently than WT cells, and exhibited transient events of cell body waggle (where the cell body rocks back and forth in place) more frequently than WT. As with flagellar beat frequency, this phenotype was more severe in *cpc1-2* than *fap246-1*. We describe the new data on page 7 of the revised text.

Second, in their proteomics work the authors note reductions in two proteins that were previously identified as part of the C1b complex, but that also occur elsewhere in cilia, HSP70 and enolase. Once again, without a direct comparison to cpc1, the authors cannot tell whether the reductions in the ratios of these proteins in fap42 vs wild type axonemes (35% reduction for enolase, 60% for HSP70A) represents a loss of the entire amount of each protein normally present in the C1b complex. If the fap42 mutation completely disrupts the association of these two proteins with C1b, then some of the missing structure in the cryoEM averages should correspond to these proteins. This mass is not trivial, as the total of the two proteins is 123 kDa, similar to that of FAP246 (120 kDa). At the least the authors should note their inability to rule out the possibility that some of the missing structure in their fap42 tomograms could represent these proteins. Perhaps this represents the difference between the estimated total of 550 kDa and the observed total of 450 kDa for the pillar (p. 12, last paragraph)?

We thank the reviewer for this comment and agree. Therefore, we have added two sentences as suggested on page 9. Concerning the somewhat lower size estimate of ~450 kDa for the pillar, we have added some possible explanations on page 16.

In addition, could the reduction in swimming speed could be due mostly to reductions in ATP concentrations in the ciliary compartment, from loss of enolase activity, as suggested by Mitchell et al., 2005? These possibilities should be discussed in the interpretation of the presented data.

We thank the reviewer for pointing this out. We have added a brief discussion about this on page 17.

Third, because *Chlamydomonas* is a model organism and has provided groundbreaking advances in ciliary ultrastructure, the results here are important whether the proteins under analysis are broadly distributed phylogenetically or not. However, it would provide the reader with a greater ability to follow the relative importance of these results if some of the information on phylogeny, previously so carefully analyzed by the Witman lab, and on the relative conservation of structure, previously analyzed by the Nicastro lab, were referenced here. In particular, the bracket structure is completely missing in sea urchin (Carbajal-Gonzalez et al., 2013), which is not surprising as the FAP246 and FAP413 proteins assigned to this structure here are limited to bikonts and green algae, respectively, and so are completely missing in metazoa (Zhao et al., 2020). More surprising and also worthy of comment, the beam structure is here identified with the FAP42 protein. Although similar structures occur in sea urchin (Carbajal-Gonzalez et al), FAP42-related genes have a quite limited distribution among Cryptophyte and Haptophyte lineages and do not appear in metazoa (Zhao et al). The discussion should be expanded to include some information on the applicability of these data to other organisms.

We thank the reviewer for mentioning these interesting points. As suggested, we have now discussed this in the revised manuscript on page 15.

Minor points:

1. Table 1 indicates proteins that are "significantly" reduced in bold print, but no criteria for significance were described.

We thank the reviewer for pointing out this oversight. We have now added the following information to Table 1:

" [a] Unique peptide numbers that are missing or significantly reduced with mutant/WT ratios <0.2

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(or number of peptides <0.5 of WT) are highlighted in bold."

2. p. 10, in describing the results presented in Fig. 2S, class3, "base" should be "tip".

We thank the reviewer for pointing out this error, which has been corrected in the revised manuscript.

3. Discussion, p. 14, in discussing the effects of their mutations on remaining C1b and C1f structures, the authors say "All these structures have a 16 nm periodicity…". However, C1f clearly has a 32 nm periodicity, with two different structural shapes alternating along the C1 microtubule (as seen, for example, in Fig. 2 D and H, Fig. 3 E and J and Fig. 4 D and H, and Fig. S2 B, D and F).

We thank the reviewer for pointing out this error. We have revised the sentence on page 15 as follows:

"The C1b-projection has a 16-nm periodicity along the C1-microtubule, whereas the C1f-projection contains structural features that repeat with 32-nm periodicity, similar to the neighboring C1d-projection (Movie 1) …"

4. Discussion, p. 18, the authors state that the C2b projection in fap42 did not show obvious positional instability or reduction, but C2b clearly appears to have lost density in *fap42* as seen in Fig. S2 E.

We appreciate the reviewer's careful observation. We agree with the reviewer, but initially attributed the apparently weaker density to the fact that the positional relationship between the C1 and C2 microtubule is somewhat flexible in *Chlamydomonas* and because the subtomogram alignment typically favors the C1 microtubule with its prominent projections; as a result, the C2 microtubule and its projections usually appear blurred unless local alignment is applied (Carbajal-González et al., 2013). We refined the local alignment and reanalyzed the C2b projection using classification. We found the density of the C2b projection is indeed reduced to various degrees in the C1b mutants. We have changed the text accordingly throughout the text (e.g. on pages 11-13, 16 and 18), added a supplementary figure S3 K-P with the C2b classification result, and expanded the field of view for the cross-sectional cartoons of summary figure 5C-F so that the cartoons now include C2b to reflect this new finding.
The above finding raises the issue of why the cryo-ET average and classification show a reduction of the C2b projection, when our mass spec analysis of fap42 axonemes does not report a similar reduction in hydin, which so far is the only protein proposed to be located in the C2b projection. We discussed possible reasons on page 17 (top).

5. The extent of the dark blue coloration of the C1b pillar in isosurface images is inconsistent, extending to the C1 microtubule surface in the main text figures, but not in Fig. S2 or in movies S1 or S4.

We thank the reviewer for pointing out this inconsistency. This has been corrected in all revised figures and movies.

6. In the legend to Fig. S2, fap42 should be set in Italics.

We thank the reviewer, and the font has been corrected in the revised manuscript as suggested.

Reviewer #2: *Advance Summary and Potential Significance to Field*:

Central pair apparatus plays important regulatory roles in ciliary beat. Mutations of central pair proteins are linked to important human diseases. The central pair structure is huge, extremely complicated and among one of the most challenging targets for modern microscopy. Lacking a detailed architecture of central pair has largely limited the current understanding of its regulatory roles. Any progress that can provide a clearer picture of this gigantic machinery will improve our understanding of ciliary beating. Cai et al. reported their new results on the architecture of a sub-complex localized in C1b. The methods, analysis and manuscript are of good quality in general.

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We thank the reviewer for this positive assessment.

Reviewer 2 Comments for the Author: Major issues:
(1) A most critical issue is that the authors lack a strong proof for the currently proposed localization of FAP413. The only evidence that helped the author to reach the conclusion is the mass spectrometry results (Table 1), which show that FAP413 is almost lost in the fap42 or fap246 mutants. It does not necessarily support that FAP413 must interact FAP42 or FAP246 to form the triple-complex that the author referred to as FAP246-FAP413-FAP42. Unlike fap42 or fap246, of which the authors did a very good job on the structural characterization, the fap413 mutant was not even tested. It is normal that lacking a certain subunit in an intricate protein complex will affect many of its neighbors. Not only its neighbors, sometimes other proteins that seem to be far away can also be remotely affected. I therefore suspect that there is a possibility that fap413 is localized in a different region unless the authors provide a much stronger evidence. The concern applies to other proteins that the authors had discussed but just not stated as their conclusions in this manuscript.

We agree with the reviewer and therefore, we have now performed cryo-electron tomography (combined with MS/MS analysis) of the flagella of a *fap413* mutant that has become available in the *Chlamydomonas* CLiP mutant library. The mass-spectrometry analysis confirmed the loss of FAP413 in the *fap413* axonemes (*fap413*/WT ratio = 0.00) (revised Table 1). Subtomogram averaging and classification analyses revealed a partial loss of density in the inner bracket domain, close to the position proposed in our original manuscript.
The new data, however, provide a more refined picture of the C1b architecture. We have added the *fap413* results throughout the manuscript, including Figs. 2 I-K, 5F, S3A-D, M and N. Despite the complete loss of FAP413 (as shown by our MS/MS), we found only a partial loss of the bracket density. There are several possible explanations for this observation, which we describe on page 11-12.

Other comments:
(2) In the current, as well as previous studies, the authors estimated the mass of several local regions of the central pair complex. I appreciate the authors' efforts, but one thing that has not been well discussed is the stoichiometry of each protein. This can sometimes affect the localization of the proteins. I am not doubting the current model, but rather suggest the authors at least provide their own data on this issue or previous evidence or some kind of analysis on different possibilities to propose a most likely model.

We appreciate the reviewer's suggestion and agree that the accuracy of mass estimations based on cryo-ET averages can make stoichiometry interpretations difficult, especially if the sizes of subunits are close to the possible error margin of these estimates (which is thought to be about +/- 30 kDa). However, in the present study the large predicted molecular mass of most of the studied subunits is of assistance. Our molecular weight estimates are consistent with the stoichiometry of FAP246:FAP413:FAP42 being 1:1:1. However, we cannot exclude the possibility that one or more copies of other, smaller proteins (such as FAP174 and/or FAP380) might also locate to C1b, C1f or C2b. We are briefly discussing this in the revised text on page 16.

(3) As a research article, the most important result of this manuscript is no doubt the localization and possible architecture of the protein complexes themselves. All the rest of discussions, possible interactions, regulatory mechanisms and sensory roles etc. etc. are entirely based on the reliability of the structure itself. Yet, the authors discussed many things based on their limited model, far beyond what the data can support. This is risky and may affect the manuscript if the basis itself has a problem. So, my suggestion is that the authors substantially weaken some of the discussions but solidify their structural basis first. It is indeed the result itself that matters in the long term. To discuss so much without a solid basis makes me a bit uncomfortable about the manuscript. For example, the author claim that the C1b may be involved in nucleotide cycles. This hypothesis itself makes sense and I do not necessarily disagree with the authors. The issue is why it is related to this manuscript if the authors did not provide any of their own evidence. Without the cryo-ET structure, we can still make hypothesis on possible roles of FAP42 and FAP246 without determining the cryo-ET structure?
We partially agree with the reviewer. We believe that our structural basis is solid (especially as we have now imaged also the *fap413* mutant). We also think that discussing possible roles of the C1b

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proteins – including results from previously published studies - is important in the light of our new structural and compositional data. For example our data contribute significantly to the discussion about the mechano-sensory role of the CA, resulting in our model that the C1b projection may resist compression force during axonemal bending, which is important for signal-transmission to regulate dynein activity and ciliary motility. However, we agree with the reviewer that the title of this section was too speculative, and we have revised the header to better reflect the scope of the discussion (page 17) to:

"C1b proteins contribute to the regulation of ciliary beating"

In regards to the discussion of nucleotide cycles, please note that Reviewer 1 asked us to discuss if the reduction in swimming speed could be due to loss of enolase activity. Given that the C1b projection contains proteins known to be involved in nucleotide metabolism, we feel that this is appropriate to address this in the context of our new mass spec data.

The author stated that 'the FAP246-FAP413-FAP42 subcomplex is part of a large interconnected CA network" and "the FAP246-FAP413-FAP42 subcomplex provides mechanical support". Again, the issue is: does the FAP246-FAP413-FAP42 ever exist? If not, why the authors discuss about all these possible roles.

We hope that the opinion of the reviewer has somewhat changed in light of the additional *fap413* data. We believe that our results (cryo-ET and MS/MS) clearly show structural proximity and interdependence between these three subunits, i.e. loss of FAP246 results in loss of FAP413 (but not vice versa), and loss of FAP42 results in loss of FAP246, which strongly suggests a (MDa-sized) interconnected C1b network. No changes have been made.

(4) I suggest the authors slightly soften some of their statements and compact some of the unrelated details, but focus a bit more on the most important point and provide sufficient evidence. For example, in the section "FAP246 is localized to the C1b projection and forms a complex with FAP413". I was trying to find any possible evidence that FAP246 and FAP413 form a complex, but unfortunately failed to understand how they reached this conclusion. Other details are good, but not essential for the main conclusion of their sub-title.

Please see our response to the previous point. Concerning discussions that were not directly related to our structural data and complex organization, we have slightly softened/reduced our discussion as suggested by the reviewer.

(5) I have a minor suggestion on the movie S2 and S3. Could it be possible to obtain more classes and make smoother movies?

We appreciate the suggestion, but unfortunately, splitting the data into more classes/intermediate states would reduce the number of particles in each class, resulting in very noisy class averages. In addition, the major points of the movies is not to show (or even imply) a particular motion, but rather by "flipping" between the classes we believe that the positional flexibility becomes a bit easier to appreciate than in figure panels side by side. No changes have been made to the movies.

References mentioned:

Mitchell, B. F., Pedersen, L. B., Feely, M., Rosenbaum, J. L. and Mitchell, D. R. (2005). ATP production in Chlamydomonas reinhardtii flagella by glycolytic enzymes. *Mol Biol Cell* 16, 4509-18.

Mitchell, D. R. and Sale, W. S. (1999). Characterization of a Chlamydomonas insertional mutant that disrupts flagellar central pair microtubule-associated structures. *J Cell Biol* 144, 293-304.

Zhang, H. and Mitchell, D. R. (2004). Cpc1, a Chlamydomonas central pair protein with an adenylate kinase domain. *J Cell Sci* 117, 4179-88.

Zhao, L., Hou, Y., Picariello, T., Craige, B. and Witman, G. B. (2019). Proteome of the central apparatus of a ciliary axoneme. *J Cell Biol* 218, 2051-2070.

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Second decision letter

MS ID#: JOCES/2020/254227

MS TITLE: Structural organization of the C1b projection within the ciliary central apparatus

AUTHORS: Kai Cai, Yanhe Zhao, Lei Zhao, Nhan Phan, Yuqing Hou, Xi Cheng, George Witman, and Daniela Nicastro
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. Please make text changes by following Reviewer #1's comments.

Reviewer 1

Advance summary and potential significance to field

This paper provides important advances to our understanding of these incredibly complex and challenging ciliary structures, and presents data that range from motility to biochemistry to high-resotution EM cryo-tomography. Anyone working on this structure and its mechanisms of motility regulation will need to reference this detailed work.

This revised manuscript includes extensive responses to the initial reviewer suggestions and comments. The authors should be commended for their serious interest in getting the science right, even at the expense of the additional time and experimentation needed to respond to the reviews.

Comments for the author

Three minor editorial changes are needed to provide missing words or to complete partial sentences:
p. 5, last paragraph, the sentence beginning "The C1b-projection..." is an incomplete sentence fragment.

p. 14, third line from the bottom, the work "protein" is missing from "...a C1-microtubule protein."

p. 19, first sentence of the last paragraph, the word "radius" is missing from "...cylinder with ~40 nm radius."

Reviewer 2

Advance summary and potential significance to field

The work has signifcantly improved our understanding of the central pair apparatus. On the other hand, the authors have performed extensive structural analysis, which requires non-trival efforts due to the complexity of central pair. It is very important and worth publishing in JCS.

Comments for the author

I do not have more questions regarding the results themselves. The revised version is much improved. The results are convincing, and the quality of data analysis is very good. Therefore, I highly recommend publication of the paper in JCS.

My only concern is that the authors discussed too many things that still need further investigation. I don't necessarily doubt the possibilities that the authors have raised. I also fully understand that the authors intended to interpret from their current results as much as possible, but the issue is that not all of them are directly related to the finding of the paper itself. My suggestion is that the

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authors may think about removing some of their speculations in the discussion sections and/or make the tone of their speculations a bit weaker. Just for example, "Taken together, the results suggest that the C1b and C2b-projections function in concert to regulate the timing of dynein activity". I felt the current data cannot support the following statement at all. From central pair components to dynein activity, there is still a very long journey for possibly years of follow-up research. It will not hurt the scientific contributions of the paper at all if the speculations can be limited to a reasonable level.

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