JCB

CEP104/FAP256 and associated cap complex maintain stability of the ciliary tip

Thibault Legal, Mireya Parra, Max Tong, Corbin Black, Ewa Joachimiak, Melissa Valente-Paterno, Karl Lechtreck, Jacek Gaertig, and Khanh Huy Bui

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March 23, 2023

Re: JCB manuscript #202301129

Prof. Khanh Huy Bui
McGill University
Anatomy and Cell Biology
3640 rue University
Montreal, Quebec H3A 0C7
Canada

Dear Prof. Bui,

Thank you for submitting your manuscript entitled "Molecular architecture of the ciliary tip revealed by cryo-electron tomography." The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers appreciate that the work reveals new and important details into the structural organization of the ciliary tip. Their detailed comments are aimed at improving data presentation and discussion as well as requests for additional analyses of existing data but do not require new experiments.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

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Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to

find an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Maxence Nachury, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

General assessment

In this manuscript, Legal et al. revealed detailed structural features of the ciliary tip using cryo-ET of Tetrahymena cilia. Ciliary tips have distinct structures from the rest of the ciliary axoneme, which is essential for ciliogenesis, ciliary length regulation, and proper ciliary motilities. Despite their importance, most of the knowledge about ciliary tips comes from conventional electron microscopic observations, which limits our understanding of the ciliary tip's native structures.

An important advancement made in this study is the detailed description of the ciliary tip structures. At the tip, the B-tubules of doublet microtubules are absent, leaving only singlet A-tubules, which results in a narrowing of the ciliary diameter. Additionally, filamentous "plugs" extend from the microtubule cap to the inside of the microtubules. The ends of central-pair microtubules are covered by protein densities called "the cap complexes", which are intertwined. Using subtomographic averaging, it was revealed that the central-pair structures at the ciliary tip undergo a transformation, with the general C1- or C2-projections being replaced by an 8 nm repeat of spike proteins that attach to both C1- and C2-microtubules. Furthermore, newly discovered linker structures stabilize the ciliary tip structures by connecting either singlet A-tubules or a singlet A-tubule and the ciliary membrane.

The study also made progress in the functional analysis of CEP104/FAP256, a known protein enriched in the ciliary tip. The loss of cap complexes and the abnormal bending of singlet A-tubules observed in CEP104/FAP256 mutants imply that CEP104/FAP256 is involved in the formation and stability of ciliary tip structures. The mass spectroscopic analysis of CEP104/FAP256 mutants identified six novel potential candidates for ciliary tip components.

In conclusion, this study offers significant insights into comprehending the structure of the ciliary tip, which can potentially serve as a point of reference for analyzing similar structures in other species. Identifying potential components of the ciliary tip can also enhance our understanding of its formation and functionality. However, certain aspects of the results and discussion require further clarification and improvement, as essential information necessary for comprehending the authors' models (Fig. 7 and Fig. S6B) is either absent or unverified.

Major comment

(1) General information and some important phenotypes of FAP256 mutant are not provided.

(1)-1
The origin of FAP256A/B mutant is not clarified in this manuscript. Is this mutant the same as the one generated by Louka et al., 2018? If FAP256A/B mutants are created in this study, the procedure and the mutation validation should be shown in the Materials and Methods section.

(1)-2
The phenotypes of FAP256 mutant is not fully described.
In WT, the following phenotypes were quantified:

(a) length of A-tubule (Fig. 1C and D),
(b) distance between A-tubule singlets (Fig. 3F),
(c) distance between C1 and C2 (Fig. 4C),
(d) length of CP (Fig. 5D), and
(e) twist of CP (Fig. S4D).
Although the data of (d) (Fig. 5D) and (e) (Fig. S5D) are shown for FAP256 mutant, quantified data of (a), (b), and (c) are also required. Especially the data of (b) are important to discuss the function of FAP256, as authors propose that "FAP256 contributes to stabilising the ciliary tip microtubules by preventing sliding and bending of these microtubules (Figure 7)" (lines 449-452).

(1)-3
The method to measure the CP length (Fig. 5D) is not clarified.
In WT, the authors mentioned that "the ends of CP microtubules were difficult to visualise because of the ciliary cap" (lines 162-163). The criteria to decide the ends of the CP microtubule needs to be described in Materials and Methods. Moreover, in the FAP256 mutant, authors found that "C1 and C2 had different lengths" (lines 285-286). Which CP microtubule is used for the length measurement of mutant CP?

(1)-4
Authors interpreted the CP defects in the FAP256 mutant as the "sliding of one of the CP microtubules compared to the other" (line 438). However, the shortage of the tip CP length (Fig. 5D) suggests the insufficient formation or abnormal disassembly of the tip CP microtubules in the mutant, which can also explain the different lengths of the C1-tip and C2-tip. A more detailed explanation is required to understand the proposed model in Fig. 7, especially the relationship between the CP defects and the sliding of CP microtubules in the mutant.

(2) Molecular interactions of FAP256, CCDC81, and microtubule are not verified.

(2)-1
Alignment data of the N-terminal domain of CCDC81with that of IJ34 is not shown, although the authors mentioned it in lines 314-316: "The N-terminal CCDC81 domain aligns well with that of IJ34, another CCDC81 homolog, recently identified as a B-tubule MIP binding on the surface of the A-tubule at the inner junction (Kubo, Black, et al. 2022)."

(2)-2
Fig. S6B is misleading because this manuscript does not show any direct interactions between CCDC81 and microtubule or between FAP256 and CCDC81. Especially the interaction between FAP256 and CCDC81 is over-discussed. Additional biochemical analyses (immunoprecipitation, co-pellet assay, yeast two-hybrid analysis, etc.) are required to reveal these interactions. Moreover, the localization of CCDC81 in the ciliary tip region needs to be verified.

(3) Data of MS analysis needs to be sufficiently described.

(3)-1
Raw results of MS analysis should be provided as supplemental information.

(3)-2
In this manuscript, the authors listed only the proteins missing in mutant cilia (Table 1) or proteins showing a fourfold reduction in mutant cilia (Table S3). However, proteins increased in mutant cilia (Figure 6A, right side) are also essential to think about the functions of the ciliary tip and CEP104/FAP256, as the ciliary tip works as the zone of ciliary growth. It would be good to add a list of proteins significantly increased in mutant cilia, with each human homolog name and known function like Table 1.

-

Minor comments

>line 36: (reviewed in (Satir and Christensen 2007)
Remove the second "(".

>line 36: (Satir and Christensen 2007).
>line 39: (Reiter and Leroux 2017).
>line 44: (Ichikawa, Liu et al. 2017). and so on.
Change the citation style. (Satir and Christensen, 2007), (Reiter and Leroux, 2017), (Ichikawa et al., 2017), etc.

>line 96: CHE-12/Crescerin, CEP104/FAP256
Add "and". "CHE-12/Crescerin and CEP104/FAP256"

>line 129: cap complex
Both "cap complex" and "tip complex" is used in this manuscript.
Unify the usage of these words. "Cap complex" is more used than "tip complex".

>line 138: Within the tip region, the projections of the CP are replaced by a characteristic short
>line 139: spike protein which repeats every 8 nm.
The spike structures should be indicated in Fig. 1A or 1B.

>line 145: we measured the A-tubule starting zone to span over 282.6
>line 146: {plus minus} 61.8 nm. Similarly, the A-tubule ending zone showed substantial variation between
>line 147: cilia with an average of 374.1 {plus minus} 277.4 nm.
In Fig. 1C, the length of the A-tubule starting zone is ~374 nm, while the length of the A-tubule ending zone is ~283 nm. Which description is true?

>line 150: Finally, some B-
>line 151: tubules separate from the inner junction (Fig. 1E, S1B).
In Fig. 1E-section(c), both junctions of doublet microtubules are indicated as "Outer junction".
The junction facing the center should be the "inner junction".

>line 155: summarised in a model figure and a movie (Fig. 2A, Movie S1).
There is no legend for Movie S1 in this manuscript.

>line 185: The resulting average has a resolution of around 100 Å
In Table S2, the resolution of the tip complex structure is 129.5 Å.
It would be more accurate to change the description of the resolution to "around 130 Å" or "even worse than 100 Å".

>line 216: When comparing our
>line 217: structure to other species, we found that the Tetrahymena CP is most similar to sea
>line 218: urchin CP (Carbajal-González, Heuser et al. 2013), especially when comparing C1d
>line 219: and C1c projections (Fig. S4C).
To compare these structures, add the separated images of Tetrahymena and sea urchin CPs in Fig. S4C, in addition to the superimposed image of CPs.

>line 243: The
>line 244: resolution we obtained was about 9 Å which allowed us to distinguish between α- and
>line 245: β-tubulin subunits (Fig. 4D, S4F-G, Table S2).
The resolution seems to be significantly different between tubulins and MAPs. It would be good to add a local resolution map in Fig. S4.

>line 273: The filaments going inside the CP microtubules were still
>line 274: present (Fig. 5B, S5A, Fig. 2B).
>line 277: we saw that the CP microtubules end with slightly curved protofilaments that contact
>line 278: the membrane directly (Fig. 5B and C).

Images in Fig. 5B and 5C have an artifact of horizontal stripes. Replace the images with better ones.

>line 316: However, when
>line 317: overlaid with tubulin, parts of the microtubule-binding domain of IJ34 are missing in
>line 318: CCDC81.
The origin of the structural data of IJ34 and FAP256 needs to be clarified.
The method to overlay the IJ34 and CCDC81 structures on tubulin should be provided in Materials and Methods.

>line 327: In addition, sperm-tail PG-rich
>line 328: repeat protein and tubulin-tyrosine ligase were absent although not significant.
The corresponding protein names for "sperm-tail PG-rich repeat protein" and "tubulin-tyrosine ligase" are not described in this manuscript.

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, the authors utilized cryo-electron tomography to characterize the 3D structures of the motile cilium tip region of wild type and FAP256A/B-knockout tetrahymena. The results not only confirmed previously reported overall architecture of

cilium tip region, but also provided a high-quality structural map for the central pair complexes near the tip which is different from that in the main axoneme. In addition, the manuscript identified 6 proteins that were missed from the FAP256A/B knockout tetrahymena by mass-spec study. There are some interesting new structural information in the manuscript, particularly on the central-pair-microtubule associated structural features that should be published. However, the overall design of the manuscript and writing tactic should be modified to significantly improve the merit of the manuscript.

• The first major result of the manuscript states as "The ciliary tip of Tetrahymena is structurally different from the rest of the axoneme". There is little new information offered by the statement itself as this is known as early as 1980 (Dentler 1980a, 1980b). The overall architecture of the motile cilia tip region are mainly presented in Figs 1A-1C, which is found similar to that reported previously by Reynolds et al (2018) using cryo-electron tomography(Reynolds et al. 2018) (see the Figure 4 and 5 of Reynolds 2018). These published works are closely related to the data presented in this manuscript. However, they are not properly acknowledged or discussed, neither in the result section nor in related discussion and the Instruction section. A good quality manuscript should clarify what is known (data and idea) so that what is new (either data or idea, or both) may be emphasized on.
• The second major result of the manuscript states "The tip microtubules interact with many proteins". Again, this subtitle itself offers little new information, as Dentler and colleagues have published much data back in 1980S regarding structural components attached to the tip region of the microtubules. A subtitle focusing on new information, idea or discovery is recommended.
• Most new results of this manuscript are presented in the section subtitled "The tip CP microtubules are strongly linked to each other". This section should be emphasized on and significantly expanded to improve the manuscript quality. The authors identified the structural difference of the central pair microtubules near the tip comparing to that in the main axoneme. Through sub-volume averaging, the structural map of CP near the tip has reached resolutions ranging from 8.8Å to 10.5Å for different part of the density maps, resolving secondary structural features. The map quality is good enough for protein identification and map fitting by known or predicted protein structures. This can be easily carried out in refence of the recently published high-resolution structure of central pair complex (Han et al. 2022; Gui et al. 2022) and alpha-fold predicted structures of possible candidate proteins.
• Some of the statements in the manuscript are not accurate. For example, Line 181 states "this is the first observation of IFT trains on the A-tubules in motile cilia". This is incorrect. As the manuscript also noted, Stepanek and Pigino studied IFT by CLEM and visualized trains on both A- and B-tubules, respectively (Stepanek and Pigino 2016). In addition, this sentence offers little biological or structural information and does not help to keep the manuscript concise.
• The manuscript also reports the result of subvolume averaging of the tip cap and its associated central microtubules. The interaction between tip cap and microtubules is not rigid. This can be seen in some of the images in this manuscript that agree with other published data. Therefore, it is not a surprising that the resultant map of subvolume averaging gives a low resolution which does not offer reliable structural details. With the unsatisfying map quality, the statement of Line 185 becomes kind of exaggerated that the "resulting average..... reveals that the CP cap is asymmetrical". I would suggest to frankly state that "The resulting averaged map has a resolution of around 100 Å. The unsatisfying result reflects the structural character that the tip cap is not rigidly attached to the tip of the two central microtubules (Fig. 2G, S2F, table S2)."
• Line 332, "we identified one protein 332 as membrane-binding (I7MKU9, Fig. 6B) and two proteins as microtubule-binding". Since these are not solid conclusions, but hypotheses based on structural predictions, I suggest change the wording to "we propose one protein 332 as membrane-binding (I7MKU9, Fig. 6B) and two proteins as microtubule-binding". The paragraph contains mostly discussion and speculation on binding partners and potential functions, and these contents should be moved to discussion section.

Reference:
Dentler, W. L. 1980a. 'Microtubule-membrane interactions in cilia. I. Isolation and characterization of ciliary membranes from Tetrahymena pyriformis', J Cell Biol, 84: 364-80.
Dentler, W. L. 1980b. 'Structures linking the tips of ciliary and flagellar microtubules to the membrane', J Cell Sci, 42: 207-20.
Gui, M., X. Wang, S. K. Dutcher, A. Brown, and R. Zhang. 2022. 'Ciliary central apparatus structure reveals mechanisms of microtubule patterning', Nat Struct Mol Biol, 29: 483-92.
Han, L., Q. Rao, R. Yang, Y. Wang, P. Chai, Y. Xiong, and K. Zhang. 2022. 'Cryo-EM structure of an active central apparatus', Nat Struct Mol Biol, 29: 472-82.
Reynolds, M. J., T. Phetruen, R. L. Fisher, K. Chen, B. T. Pentecost, G. Gomez, P. Ounjai, and H. Sui. 2018. 'The Developmental Process of the Growing Motile Ciliary Tip Region', Sci Rep, 8: 7977.
Stepanek, L., and G. Pigino. 2016. 'Microtubule doublets are double-track railways for intraflagellar transport trains', Science, 352: 721-4.

Reviewer #3 (Comments to the Authors (Required)):

The manuscript by Legal et al. attempts to analyze the ciliary tip in Tetrahymena using cryo-electron tomography. The ciliary tip is a specialized compartment where the outer doublet microtubules transition to A-singlet microtubules and are then capped by structures that linked the A-tubules to one another and the ciliary membrane. In motile axonemes, the ciliary tip is also the site where the two singlet microtubules of the central pair complex are capped by another structure that links the CP microtubules to

the ciliary membrane. This region has been the subject of intense interest as the site of tubulin addition during ciliary assembly, a potential site for the regulated remodeling of the IFT particles and IFT motors, and a site for the coordination of ciliary signaling pathways. This region has previously been studied by thin section transmission electron microscopy and by high resolution light microscopy. Recent studies of ciliary structure using both cryoET and single particle cryoEM have primarily focused on the proximal and medial regions of the axonemal doublet microtubules. In this study, the authors are capitalizing on previous work from the Gaertig lab that identified CHE-12/crescerin and ARMC9 as potential regulators of doublet microtubule assembly and FAP256 as a component of the central pair cap structure and regulator of A-tubule elongation.

The key findings of the present study are as follows:

• At the tip region, the projections of the CP microtubules are replaced by a short spike protein.
• Not all doublet microtubules become singlet microtubules at the same level.
• The A-tubules are capped by a filamentous plug that connects to both the CP and membrane.
• The A-tubules contain MIPs (microtubule inner proteins) that differ from MIPs found in doublet MTs.
• The CP microtubules also contain MIPs, some attached to the MT wall and others inside the lumen.
• Variable densities appear to link the A-singlets to one another and to the membrane.
• The tips of the two CP microtubules are strongly linked to one another.

For the most part the resolution of the tomograms is not sufficient to identify the individual proteins.
However, the authors have compared the structure of the ciliary tip in WT cells with the ciliary tip in FAP256 K/O cells and found the following.
• The FAP256 KO mutants lack the CP cap but the filaments inside the CP microtubules are still present.
• The lengths of the CP microtubules are more variable in the mutant compared to WT cells.
• Overall, the tip region of the CP microtubules is shorter in the mutant.
• The structure of the A-tubule singlets is not significantly different, except that they tend to curve toward the CP microtubules in the mutant.

To identify components of the CP cap structure, the authors compared the protein composition of WT and FAP256 KO flagella by mass spectrometry.
• Their results are listed in Table 1 and Supplemental Table 3.
• The proteins listed in the two tables are different, and it is unclear why this is the case.
• No information was given about the total number of proteins identified by their screen and how they determined which proteins were significantly different in the mutant.
• A model showing the proposed locations of the missing proteins in WT cilia is shown in Figure 6.

As I am not an expert on the details of the tomography, I will confine my comments to where I think the authors could improve the clarity of their data and its presentation.

1) The authors should summarize their findings on the distal structures of the A-tubules and CP MTs in a Table, indicating how they differ from similar structures in the proximal and medial regions of the axoneme.
2) They should also use a diagram summarize how these structures are altered in the FAP256 K/O strain.
3) They need to provide more information about their mass spectrometry data.
• How many proteins were identified in the WT and mutant samples?
• How many replicates were analyzed?
• How many peptides per protein?
• What is the % sequence coverage?
• How do they determine that a given protein is significantly reduced in the mutant?
• What is the meaning of the proteins listed in Table 1 and Supplemental Table 3 and why are they completely different?
• When they refer to the human homologs, how significant are these homologies?

Khanh Huy Bui, Ph.D.

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2023-07-13

Dear Dr. Nachury,

Please find the revision of our manuscript titled ***"Molecular architecture of the ciliary tip revealed by cryo-electron tomography"***.

In the revised manuscript, we made a lot of efforts to address the comments from the reviewers, especially to confirm whether CCDC81 is a cap-complex component. During the last three months and a half, we constructed the tagged CCDC81 to confirm its localization. Our new result of super-resolution structured illumination microscopy indicated that CCDC81 localizes to the tip of the cilium. We can now confirm that CCDC81 is a novel tip complex protein. In addition, we have carefully addressed everything else suggested by the reviewers and improved the readability of the manuscript.

The point-to-point answers to the reviewers are included.

Kind regards,

Khanh Huy Bui

I would like to thank all the reviewers for their constructive comments. We have revised the paper to address the concerns of the reviewers. In summary, here is what we included in the revised version.

- We carried out data analysis and improved the clarity of our writing and the figures according to the reviewers' suggestion and our own judgement.
- To confirm CCDC81 is a good candidate for the cap complex, we generated a *Tetrahymena* strain with a GFP-mNeonGreen tag. We performed both super-resolution structured illumination microscopy (SR-SIM) and Total internal reflection fluorescence (TIRF) on the strains. Both techniques show that CCDC81 can be located at the distal tip (Figure 6). These experiments were carried out by Mireya Parra and Karl Lechtrek who should be added to the authors' list as well as Ewa Joachimiak.
- To increase the transparency of our research work, we deposited the cryo-EM data to the EMDB and the MS data to PRIDE. The mass spectrometry data can be accessed with username reviewer_pxd042582@ebi.ac.uk and password: QRbLDczR

The newly added text is highlighted in the manuscript for easy viewing.

Below are our point-to-point answers to the reviewers.

Reviewer #1 (Comments to the Authors (Required)):

General assessment

In this manuscript, Legal et al. revealed detailed structural features of the ciliary tip using cryo-ET of Tetrahymena cilia. Ciliary tips have distinct structures from the rest of the ciliary axoneme, which is essential for ciliogenesis, ciliary length regulation, and proper ciliary motilities. Despite their importance, most of the knowledge about ciliary tips comes from conventional electron microscopic observations, which limits our understanding of the ciliary tip's native structures.

An important advancement made in this study is the detailed description of the ciliary tip structures. At the tip, the B-tubules of doublet microtubules are absent, leaving only singlet A-tubules, which results in a narrowing of the ciliary diameter. Additionally, filamentous "plugs" extend from the microtubule cap to the inside of the microtubules. The ends of central-pair microtubules are covered by protein densities called "the cap complexes", which are intertwined. Using subtomographic averaging, it was revealed that the central-pair structures at the ciliary tip undergo a transformation, with the general C1- or C2-projections being replaced by an 8 nm repeat of spike proteins that attach to both C1- and C2-microtubules. Furthermore, newly discovered linker structures stabilize the ciliary tip structures by connecting either singlet A-tubules or a singlet A-tubule and the ciliary membrane.

The study also made progress in the functional analysis of CEP104/FAP256, a known protein enriched in the ciliary tip. The loss of cap complexes and the abnormal bending of singlet A-tubules observed in CEP104/FAP256 mutants imply that CEP104/FAP256 is involved in the

formation and stability of ciliary tip structures. The mass spectroscopic analysis of CEP104/FAP256 mutants identified six novel potential candidates for ciliary tip components.

In conclusion, this study offers significant insights into comprehending the structure of the ciliary tip, which can potentially serve as a point of reference for analyzing similar structures in other species. Identifying potential components of the ciliary tip can also enhance our understanding of its formation and functionality. However, certain aspects of the results and discussion require further clarification and improvement, as essential information necessary for comprehending the authors' models (Fig. 7 and Fig. S6B) is either absent or unverified.

Major comment

(1) General information and some important phenotypes of FAP256 mutant are not provided.

(1)-1
The origin of FAP256A/B mutant is not clarified in this manuscript. Is this mutant the same as the one generated by Louka et al., 2018? If FAP256A/B mutants are created in this study, the procedure and the mutation validation should be shown in the Materials and Methods section.

Line 272 to 273 changed to: “We decided to use cryo-ET to study the cilia of cells lacking FAP256 (*FAP256A/B-KO*, same strain as described in Louka et al., 2018)”.

We added a sentence in the Methods section (lines 509-510): “The FAP256A/B-KO mutant was created in a previous study (Louka et al., 2018).”.

(1)-2
The phenotypes of FAP256 mutant are not fully described.
In WT, the following phenotypes were quantified:
(a) length of A-tubule (Fig. 1C and D),
(b) distance between A-tubule singlets (Fig. 3F),
(c) distance between C1 and C2 (Fig. 4C),
Although the data of (d) (Fig. 5D) and (e) (Fig. S5D) are shown for FAP256 mutant, quantified data of (a), (b), and (c) are also required. Especially the data of (b) are important to discuss the function of FAP256, as authors propose that "FAP256 contributes to stabilising the ciliary tip microtubules by preventing sliding and bending of these microtubules (Figure 7)" (lines 449-452).

The length of A-tubules, distance between A-tubules and distance between C1 and C2 in FAP256 mutants have now been added to Fig. 5F, H and E. In addition, lines 303-304 now read: “the starting and ending zones measured were 268 ± 88 nm and 194 ± 74 nm, respectively (n = 8 cilia).”.

(1)-3
The method to measure the CP length (Fig. 5D) is not clarified.
In WT, the authors mentioned that "the ends of CP microtubules were difficult to visualise because of the ciliary cap" (lines 162-163). The criteria to decide the ends of the CP microtubule needs to be described in Materials and Methods. Moreover, in the FAP256 mutant, authors found that "C1 and C2 had different lengths" (lines 285-286). Which CP microtubule is used for the length measurement of mutant CP?

We have now added a paragraph in the methods section called: “Measurements of CP and A-tubule lengths”.
Due to difficulties in visualising the ends of CP MTs in tomograms, it is likely that our measurements contain some error. However, we believe the error represents < 10nm which is < 3% of the average tip CP length in CU428. This error does not change our conclusions.

(1)-4
Authors interpreted the CP defects in the FAP256 mutant as the "sliding of one of the CP microtubules compared to the other" (line 438). However, the shortage of the tip CP length (Fig. 5D) suggests the insufficient formation or abnormal disassembly of the tip CP microtubules in the mutant, which can also explain the different lengths of the C1-tip and C2-tip. A more detailed explanation is required to understand the proposed model in Fig. 7, especially the relationship between the CP defects and the sliding of CP microtubules in the mutant.

We have made changes to the discussion.
Lines 475-479 now read: “The main two defects we observed in FAP256A/B-KO mutant cells are bending of singlets and one of the CP microtubules longer than the other (Fig. 7A). The difference in length observed between the two CP microtubules might be due to sliding of one microtubule next to the other. Alternatively, one of the two CP might disassemble abnormally or not form entirely at the tip.”.

Lines 480-483: “FAP256, along with the proteins of the cap complex might therefore prevent the sliding or the depolymerisation of CP microtubules and likely serve as an anchor for the CP microtubules to the membrane.”.

Lines 479-480: “We did not see links between A-tubules or between A-tubules and membrane in the subtomogram averages of FAP256A/B-KO singlets.”.

(2) Molecular interactions of FAP256, CCDC81, and microtubule are not verified.

(2)-1
Alignment data of the N-terminal domain of CCDC81with that of IJ34 is not shown, although the authors mentioned it in lines 314-316: "The N-terminal CCDC81 domain aligns well with that of IJ34, another CCDC81 homolog, recently identified as a B-tubule MIP binding on the surface of the A-tubule at the inner junction (Kubo, Black, et al. 2022)."

We have changed the wording, lines 335-336 were changed to: “The N-terminal CCDC81 domain overlays well with the structure of IJ34.”.
The alignment of CCDC81 and IJ34 was added to Fig. 6D.

(2)-2
Fig. S6B is misleading because this manuscript does not show any direct interactions between CCDC81 and microtubule or between FAP256 and CCDC81. Especially the interaction between FAP256 and CCDC81 is over-discussed. Additional biochemical analyses (immunoprecipitation, co-pellet assay, yeast two-hybrid analysis, etc.) are required to reveal these interactions. Moreover, the localization of CCDC81 in the ciliary tip region needs to be verified.

We made the following modifications to our manuscript:
- Fig. 6B now contains “putative microtubule-binding domain”

- Lines 342-350 now read: “The loss of CCDC81 when FAP256 is knocked out suggests these proteins might interact. We therefore tested this possibility *in silico* using different constructs and AlphaFold multimer. Some of the results pointed towards a direct interaction between the two proteins although with low confidence. We assessed the likelihood of their interaction on the same protofilament by positioning the TOG domain of FAP256 on an adjacent tubulin dimer, in a similar way to the crystal structure of Stu2 bound to tubulin (PDB: 4FFB) (Ayaz et al., 2012). When CCDC81 and IJ34 are placed on the same protofilament, their structures do not clash and are close to each other, supporting a potential interaction (Fig. 6C).”.
- We updated the methods section to include two paragraphs “Sequence alignment” and “Generation of CCDC81 and FAP256 models”.

The discussion was updated, lines 484-486 now read: “The observation that CCDC81 and FAP256 are predicted to be microtubule-binding proteins and the fact that CCDC81 is absent when FAP256 is knocked out suggests these two proteins might interact to regulate microtubule polymerisation.”.

In addition, we generated a CCDC81-GFP-mNeonGreen strain for SR-SIM and TIRF to verify the localisation of CCDC81. Both techniques show that CCDC81 localises to the entire length of some cilia and is definitely seen at the tips of some cilia (Fig. 6D, 6E, S5H, movies S2 and S3). We reflected these observations in the text (lines 351 to 362).

(3) Data of MS analysis needs to be sufficiently described.

(3)-1
Raw results of MS analysis should be provided as supplemental information.

Excel file Table S3 contains the list of the proteins identified in the three repeats of WT and FAP256A/B-KO cells. Sheet 1 contains the peptide counts for each protein, sheet 2 the percentage coverage and sheet 3 the emPAI score. Additionally, the data were deposited to PRIDE.

(3)-2
In this manuscript, the authors listed only the proteins missing in mutant cilia (Table 1) or proteins showing a fourfold reduction in mutant cilia (Table S3). However, proteins increased in mutant cilia (Figure 6A, right side) are also essential to think about the functions of the ciliary tip and CEP104/FAP256, as the ciliary tip works as the zone of ciliary growth. It would be good to add a list of proteins significantly increased in mutant cilia, with each human homolog name and known function like Table 1.

For clarity, Tables 1 and S3 were merged into a new table called Table 1. Table 2 contains the proteins increased and only present in mutant cells.

Minor comments

>line 36: (reviewed in (Satir and Christensen 2007)
Remove the second "(".

>line 36: (Satir and Christensen 2007).
>line 39: (Reiter and Leroux 2017).
>line 44: (Ichikawa, Liu et al. 2017). and so on.
Change the citation style. (Satir and Christensen, 2007), (Reiter and Leroux, 2017), (Ichikawa et al., 2017), etc.

We changed the citation style to match the required style from the Journal of Cell Biology.

>line 96: CHE-12/Crescerin, CEP104/FAP256
Add "and". "CHE-12/Crescerin and CEP104/FAP256"

We added “and” at line (now) 97.

>line 129: cap complex
Both "cap complex" and "tip complex" is used in this manuscript.
Unify the usage of these words. "Cap complex" is more used than "tip complex".

We have updated the manuscript and used only the term “cap complex”.

>line 138: Within the tip region, the projections of the CP are replaced by a characteristic short
>line 139: spike protein which repeats every 8 nm.
The spike structures should be indicated in Fig. 1A or 1B.

The spike structure is now indicated in Fig. 1B.

>line 145: we measured the A-tubule starting zone to span over 282.6
>line 146: {plus minus} 61.8 nm. Similarly, the A-tubule ending zone showed substantial variation between
>line 147: cilia with an average of 374.1 {plus minus} 277.4 nm.
In Fig. 1C, the length of the A-tubule starting zone is ~374 nm, while the length of the A-tubule ending zone is ~283 nm. Which description is true?

Fig. 1C was edited to reflect the text.

>line 150: Finally, some B-
>line 151: tubules separate from the inner junction (Fig. 1E, S1B).
In Fig. 1E-section(c), both junctions of doublet microtubules are indicated as "Outer junction". The junction facing the center should be the "inner junction".

Fig. 1E was edited accordingly.

>line 155: summarised in a model figure and a movie (Fig. 2A, Movie S1).
There is no legend for Movie S1 in this manuscript.

We added the legend for Movie S1.

>line 185: The resulting average has a resolution of around 100 Å
In Table S2, the resolution of the tip complex structure is 129.5 Å.
It would be more accurate to change the description of the resolution to "around 130 Å" or "even worse than 100 Å".

Line 185 now reads: “The resulting averaged map has a resolution of around 130 Å.”.

>line 216: When comparing our
>line 217: structure to other species, we found that the Tetrahymena CP is most similar to sea
>line 218: urchin CP (Carbajal-González, Heuser et al. 2013), especially when comparing C1d
>line 219: and C1c projections (Fig. S4C).
To compare these structures, add the separated images of Tetrahymena and sea urchin CPs in Fig. S4C, in addition to the superimposed image of CPs.

We have edited Fig. S4C accordingly.

>line 243: The
>line 244: resolution we obtained was about 9 Å which allowed us to distinguish between α- and
>line 245: β-tubulin subunits (Fig. 4D, S4F-G, Table S2).
The resolution seems to be significantly different between tubulins and MAPs. It would be good to add a local resolution map in Fig. S4.

We added the local resolution in Fig S4H.

>line 273: The filaments going inside the CP microtubules were still
>line 274: present (Fig. 5B, S5A, Fig. 2B).
>line 277: we saw that the CP microtubules end with slightly curved protofilaments that contact
>line 278: the membrane directly (Fig. 5B and C).

Images in Fig. 5B and 5C have an artifact of horizontal stripes. Replace the images with better ones.

Fig. 5B and C have been changed.

>line 316: However, when
>line 317: overlaid with tubulin, parts of the microtubule-binding domain of IJ34 are missing in
>line 318: CCDC81.
The origin of the structural data of IJ34 and FAP256 needs to be clarified.
The method to overlay the IJ34 and CCDC81 structures on tubulin should be provided in Materials and Methods.

We added a new paragraph to the methods section: “Generation of CCDC81 and FAP256 models”.

>line 327: In addition, sperm-tail PG-rich
>line 328: repeat protein and tubulin-tyrosine ligase were absent although not significant.
The corresponding protein names for "sperm-tail PG-rich repeat protein" and "tubulin-tyrosine ligase" are not described in this manuscript.

We changed the text and lines 363-367 now read: “Sperm-tail PG-rich repeat protein (Uniprot ID: Q239A2) and tubulin-tyrosine ligase (Uniprot ID: Q22BT7) were absent although not significant. Even though the human homologs of these two proteins are unclear, sperm-tail PG-rich proteins were identified as MAPs binding both inside and outside microtubules (I7M2G0 and Q24GM1).”.

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, the authors utilized cryo-electron tomography to characterize the 3D structures of the motile cilium tip region of wild type and FAP256A/B-knockout tetrahymena. The results not only confirmed previously reported overall architecture of cilium tip region, but also provided a high-quality structural map for the central pair complexes near the tip which is different from that in the main axoneme. In addition, the manuscript identified 6 proteins that were missed from the FAP256A/B knockout tetrahymena by mass-spec study. There are some interesting new structural information in the manuscript, particularly on the central-pair-microtubule associated structural features that should be published. However, the overall design of the manuscript and writing tactic should be modified to significantly improve the merit of the manuscript.

• The first major result of the manuscript states as "The ciliary tip of Tetrahymena is structurally different from the rest of the axoneme". There is little new information offered by the statement itself as this is known as early as 1980 (Dentler 1980a, 1980b). The overall architecture of the motile cilia tip region are mainly presented in Figs 1A-1C, which is found similar to that reported previously by Reynolds et al (2018) using cryo-electron tomography (Reynolds et al. 2018) (see the Figure 4 and 5 of Reynolds 2018). These published works are closely related to the data presented in this manuscript. However, they are not properly acknowledged or discussed, neither in the result section nor in related discussion and the Instruction section. A good quality manuscript should clarify what is known (data and idea) so that what is new (either data or idea, or both) may be emphasized on.

We have now included: Dentler, 1980b line 380; Dentler, 1980a line 472 and Reynolds et al., 2018 is now cited in the results at line 235 and in the discussion lines 435 and 454.
We have also changed lines 470-473: “The identity of these links remains unknown, but a previous study identified a tubulin-like protein in solubilised *Tetrahymena* membranes (Dentler, 1980a). This protein might be the one that links A-tubules to the membrane.”; and lines 380-382: “The ciliary tips of *Tetrahymena* were first studied using negative staining electron microscopy. The cap complex and A-tubule plugs were described extensively (Dentler, 1980b). However, this technique can sometimes introduce artifacts.”.

• The second major result of the manuscript states "The tip microtubules interact with many proteins". Again, this subtitle itself offers little new information, as Dentler and colleagues have published much data back in 1980S regarding structural components attached to the tip region of the microtubules. A subtitle focusing on new information, idea or discovery is recommended.

We changed the subtitles of the manuscript:
Line 124-125: Cryo-electron tomography offers three-dimensional view of the ciliary tip
Line 195: The ciliary tip singlets are maintained parallel to each other
Line 211: The tip CP has a pseudo two-fold symmetric structure
Line 268: *FAP256A/B-KO* mutants have multiple ciliary tip defects

Line 377: Length regulation of the ciliary tip
Line 426: Assembly of the CP

• Most new results of this manuscript are presented in the section subtitled "The tip CP microtubules are strongly linked to each other". This section should be emphasized on and significantly expanded to improve the manuscript quality. The authors identified the structural difference of the central pair microtubules near the tip comparing to that in the main axoneme. Through sub-volume averaging, the structural map of CP near the tip has reached resolutions ranging from 8.8Å to 10.5Å for different part of the density maps, resolving secondary structural features. The map quality is good enough for protein identification and map fitting by known or predicted protein structures. This can be easily carried out in refence of the recently published high-resolution structure of central pair complex (Han et al. 2022; Gui et al. 2022) and alpha-fold predicted structures of possible candidate proteins.

The resolution indicated refers to the highest resolution of the structure which, for the structures presented in this study, is the tubulin. All the densities binding to the microtubule have a lower resolution and do not allow us to fit proteins whose structures are known or predicted with confidence. Concerning the base CP, we have noticed significant differences compared to the CP in *Chlamydomonas* for which an atomic model is available. Obtaining an equivalent atomic model for *Tetrahymena* CP will require the resolution of our structure to be improved significantly. We have added a local resolution map of the tip CP in Fig S4H to illustrate this point.

• Some of the statements in the manuscript are not accurate. For example, Line 181 states "this is the first observation of IFT trains on the A-tubules in motile cilia". This is incorrect. As the manuscript also noted, Stepanek and Pigino studied IFT by CLEM and visualized trains on both A- and B-tubules, respectively (Stepanek and Pigino 2016). In addition, this sentence offers little biological or structural information and does not help to keep the manuscript concise.

We have now clarified this sentence:
Lines 181-182: “this is the first observation of IFT trains on the A-tubules of the singlet zone in motile cilia.”.

• The manuscript also reports the result of subvolume averaging of the tip cap and its associated central microtubules. The interaction between tip cap and microtubules is not rigid. This can be seen in some of the images in this manuscript that agree with other published data. Therefore, it is not a surprising that the resultant map of subvolume averaging gives a low resolution which does not offer reliable structural details. With the unsatisfying map quality, the statement of Line 185 becomes kind of exaggerated that the "resulting average..... reveals that the CP cap is asymmetrical". I would suggest to frankly state that "The resulting averaged map has a resolution of around 100 Å. The unsatisfying result reflects the structural character that the tip cap is not rigidly attached to the tip of the two central microtubules (Fig. 2G, S2F, table S2)."

We have modified the manuscript.
Lines 185-187 now read: “The resulting averaged map has a resolution of around 130 Å. This unsatisfying result reflects the structural character of the cap complex which is not rigidly attached to the tip of the two central microtubules (Fig. 2G, S2F, table S2).”.

• Line 332, "we identified one protein 332 as membrane-binding (I7MKU9, Fig. 6B) and two proteins as microtubule-binding". Since these are not solid conclusions, but hypotheses based on structural predictions, I suggest change the wording to "we propose one protein 332 as membrane-binding (I7MKU9, Fig. 6B) and two proteins as microtubule-binding". The paragraph contains mostly discussion and speculation on binding partners and potential functions, and these contents should be moved to discussion section.

After closer inspection, I7MKU9 might not be a membrane-binding protein so we removed this sentence. We also removed Fig. 6B. and deleted most of the paragraph.

Reference:
Dentler, W. L. 1980a. 'Microtubule-membrane interactions in cilia. I. Isolation and characterization of ciliary membranes from Tetrahymena pyriformis', J Cell Biol, 84: 364-80.
Dentler, W. L. 1980b. 'Structures linking the tips of ciliary and flagellar microtubules to the membrane', J Cell Sci, 42: 207-20.
Gui, M., X. Wang, S. K. Dutcher, A. Brown, and R. Zhang. 2022. 'Ciliary central apparatus structure reveals mechanisms of microtubule patterning', Nat Struct Mol Biol, 29: 483-92.
Han, L., Q. Rao, R. Yang, Y. Wang, P. Chai, Y. Xiong, and K. Zhang. 2022. 'Cryo-EM structure of an active central apparatus', Nat Struct Mol Biol, 29: 472-82.
Reynolds, M. J., T. Phetruen, R. L. Fisher, K. Chen, B. T. Pentecost, G. Gomez, P. Ounjai, and H. Sui. 2018. 'The Developmental Process of the Growing Motile Ciliary Tip Region', Sci Rep, 8: 7977.
Stepanek, L., and G. Pigino. 2016. 'Microtubule doublets are double-track railways for intraflagellar transport trains', Science, 352: 721-4.

Reviewer #3 (Comments to the Authors (Required)):

The manuscript by Legal et al. attempts to analyze the ciliary tip in Tetrahymena using cryo-electron tomography. The ciliary tip is a specialized compartment where the outer doublet microtubules transition to A-singlet microtubules and are then capped by structures that linked the A-tubules to one another and the ciliary membrane. In motile axonemes, the ciliary tip is also the site where the two singlet microtubules of the central pair complex are capped by another structure that links the CP microtubules to the ciliary membrane. This region has been the subject of intense interest as the site of tubulin addition during ciliary assembly, a potential site for the regulated remodeling of the IFT particles and IFT motors, and a site for the coordination of ciliary signaling pathways. This region has previously been studied by thin section transmission electron microscopy and by high resolution light microscopy. Recent studies of ciliary structure using both cryoET and single particle cryoEM have primarily focused on the proximal and medial regions of the axonemal doublet microtubules. In this study, the authors are capitalizing on previous work from the Gaertig lab that identified CHE-12/crescerin and ARMC9 as potential regulators of doublet microtubule assembly and FAP256 as a component of the central pair cap structure and regulator of A-tubule elongation.

The key findings of the present study are as follows:

• At the tip region, the projections of the CP microtubules are replaced by a short spike protein.

• Not all doublet microtubules become singlet microtubules at the same level.
• The A-tubules are capped by a filamentous plug that connects to both the CP and membrane.
• The A-tubules contain MIPs (microtubule inner proteins) that differ from MIPs found in doublet MTs.
• The CP microtubules also contain MIPs, some attached to the MT wall and others inside the lumen.
• Variable densities appear to link the A-singlets to one another and to the membrane.
• The tips of the two CP microtubules are strongly linked to one another.

For the most part the resolution of the tomograms is not sufficient to identify the individual proteins.
However, the authors have compared the structure of the ciliary tip in WT cells with the ciliary tip in FAP256 K/O cells and found the following.
• The FAP256 KO mutants lack the CP cap but the filaments inside the CP microtubules are still present.
• The lengths of the CP microtubules are more variable in the mutant compared to WT cells.
• Overall, the tip region of the CP microtubules is shorter in the mutant.
• The structure of the A-tubule singlets is not significantly different, except that they tend to curve toward the CP microtubules in the mutant.

To identify components of the CP cap structure, the authors compared the protein composition of WT and FAP256 KO flagella by mass spectrometry.
• Their results are listed in Table 1 and Supplemental Table 3.
• The proteins listed in the two tables are different, and it is unclear why this is the case.
• No information was given about the total number of proteins identified by their screen and how they determined which proteins were significantly different in the mutant.
• A model showing the proposed locations of the missing proteins in WT cilia is shown in Figure 6.

As I am not an expert on the details of the tomography, I will confine my comments to where I think the authors could improve the clarity of their data and its presentation.

1) The authors should summarize their findings on the distal structures of the A-tubules and CP MTs in a Table, indicating how they differ from similar structures in the proximal and medial regions of the axoneme.

We have summarised our findings in Table 3.

2) They should also use a diagram summarize how these structures are altered in the FAP256 K/O strain.

We have added a diagram in Fig. 7A.

3) They need to provide more information about their mass spectrometry data.
• How many proteins were identified in the WT and mutant samples?

We identified around 3000 proteins in WT and mutant samples. All the identified proteins are now listed in Table S3 and deposited to PRIDE.

• How many replicates were analyzed?

Three replicates were analysed for both WT and mutant. This has now been added to the method section (line 630).

• How many peptides per protein?
• What is the % sequence coverage?

We have now added percent coverage, emPAI score and number of peptides detected in Tables 1 and 2.
Sheets 1, 2 and 3 of Table S3 contain peptide count, percentage coverage and emPAI score for all the proteins that were identified by mass spectrometry.

• How do they determine that a given protein is significantly reduced in the mutant?

A t-test between the 3 replicates of WT and *FAP256A/B-KO* total spectra was carried out. Significant proteins had a p-value > 0.05. Now written at lines 641-643.

• What is the meaning of the proteins listed in Table 1 and Supplemental Table 3 and why are they completely different?

Table 1 contains the proteins that were not detected in the *FAP256A/B-KO* mutant while Supplemental Table 3 contains proteins that were found in mutant cells but reduced fourfold. For clarity, Table 1 and 3 were merged into Table 1.

• When they refer to the human homologs, how significant are these homologies?

We have added percentage identity from Blast for the human homologs.

August 6, 2023

RE: JCB Manuscript #202301129R

Prof. Khanh Huy Bui
McGill University
Anatomy and Cell Biology
3640 rue University
Montreal, Quebec H3A 0C7
Canada

Dear Prof. Bui,

Thank you for submitting your revised manuscript entitled "Molecular architecture of the ciliary tip revealed by cryo-electron tomography." We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below) as well as to address the remaining reviewer comments.

Reviewer #1 notes that the sequence and structural comparisons of CCDC81 and IJ32 in Figure 6 are not clear and asks to improve the presentation. Reviewer #2 requests several changes to the text and title in order to avoid giving readers the impression that this study is the first one to investigate ciliary tips using cryo-ET. Both of these issues are important and should be thoroughly addressed in the final files.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figure formatting: Articles may have up to 10 main text figures. Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add scale bars to insets in Figures 1E, 4D, and S5H.

Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. If red and green are paired for images, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Please, indicate whether 'n' refers to technical or biological replicates (i.e. number of analyzed cells, samples or animals, number of independent experiments). If independent experiments with multiple biological replicates have been performed, we recommend using distribution-reproducibility SuperPlots (please see Lord et al., JCB 2020) to better display the distribution of the entire dataset, and report statistics (such as means, error bars, and P values) that address the reproducibility of the findings.

Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) Title: The title should be concise (less than 100 characters including spaces) but accessible to a general readership. As requested by Reviewer #2 please revise the title to avoid the implication that this is the first study that looked at the structural organization of the ciliary tip by cryo-ET. Additionally, journal policy is to not mention specific methods in the title unless absolutely essential.

We suggest the following alternative titles:

"FAP256 and associated cap complex maintain stability of the ciliary tip"
or
"A cap complex maintains the architecture of the ciliary tip"

5) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

6) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate). Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary. If antibodies are not commercial, please add a reference citation if possible.

7) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

8) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

9) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures and 10 videos. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section. Please include one brief sentence per item.

10) Video legends: Should describe what is being shown, the cell type or tissue being viewed (including relevant cell treatments, concentration and duration, or transfection), the imaging method (e.g., time-lapse epifluorescence microscopy), what each color represents, how often frames were collected, the frames/second display rate, and the number of any figure that has related video stills or images.

11) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

14) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. Please note that ORCID IDs are required for all authors. At resubmission of your final files, please be sure to provide your ORCID ID and those of all co-authors.

15) Journal of Cell Biology now requires a data availability statement for all research article submissions. These statements will be published in the article directly above the Acknowledgments. The statement should address all data underlying the research presented in the manuscript. Please visit the JCB instructions for authors for guidelines and examples of statements at (https://rupress.org/jcb/pages/editorial-policies#data-availability-statement).

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you

have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Maxence Nachury, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The manuscript was revised according to the reviewers' comments. The new manuscript clearly shows the phenotypes of WT/mutant ciliary tips, the experimental procedures, and most of the author's claims in this paper.
However, Figure 6B and C that are trying to relate CCDC81 and IJ32 are hard to follow. Therefore, the figure (and related texts) requires minor revision to resolve this issue. Specific comments are as follows:

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Reviewer #3 (Comments to the Authors (Required)):

1) I summarized the key findings of the paper in my previous review.
2) In his revision, he has provided new data in support of his conclusions, particularly with respect to the localization of CCDC81
3) He has addressed my comments and criticisms about lack of clarity in the text and adding additional information about his mass spectrometry data.

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.
OK

2) Figure formatting: Articles may have up to 10 main text figures. Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add scale bars to insets in Figures 1E, 4D, and S5H.
Done

Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. If red and green are paired for images, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.
OK

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Please, indicate whether 'n' refers to technical or biological replicates (i.e. number of analyzed cells, samples or animals, number of independent experiments). If independent experiments with multiple biological replicates have been performed, we recommend using distribution-reproducibility SuperPlots (please see Lord et al., JCB 2020) to better display the distribution of the entire dataset, and report statistics (such as means, error bars, and P values) that address the reproducibility of the findings.

Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."
Added methods section.

4) Title: The title should be concise (less than 100 characters including spaces) but accessible to a general readership. As requested by Reviewer #2 please revise the title to avoid the implication that this is the first study that looked at the structural organization of the ciliary tip by cryo-ET. Additionally, journal policy is to not mention specific methods in the title unless absolutely essential.

We suggest the following alternative titles:
"FAP256 and associated cap complex maintain stability of the ciliary tip"
or
"A cap complex maintains the architecture of the ciliary tip"
Changed to “CEP104/FAP256 and associated cap complex maintain stability of the ciliary tip.”

5) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."
OK

6) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate). Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary. If antibodies are not commercial, please add a reference citation if possible.
OK

7) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).
Done

8) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.
Done

9) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures and 10 videos. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section. Please include one brief sentence per item.
Done

10) Video legends: Should describe what is being shown, the cell type or tissue being viewed (including relevant cell treatments, concentration and duration, or transfection), the imaging

method (e.g., time-lapse epifluorescence microscopy), what each color represents, how often frames were collected, the frames/second display rate, and the number of any figure that has related video stills or images.
Done

11) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.
Done

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."
Done

13) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).
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14) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. Please note that ORCID IDs are required for all authors. At resubmission of your final files, please be sure to provide your ORCID ID and those of all co-authors.
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Mireya: 0009-0002-2025-6372

15) Journal of Cell Biology now requires a data availability statement for all research article submissions. These statements will be published in the article directly above the Acknowledgments. The statement should address all data underlying the research presented in the manuscript. Please visit the JCB instructions for authors for guidelines and examples of statements at (https://rupress.org/jcb/pages/editorial-policies#data-availability-statement).
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B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

Additionally, JCB encourages authors to submit a short video summary of their work. These videos are intended to convey the main messages of the study to a non-specialist, scientific audience. Think of them as an extended version of your abstract, or a short poster presentation. We encourage first authors to present the results to increase their visibility. The videos will be shared on social media to promote your work. For more detailed guidelines and tips on preparing your video, please visit https://rupress.org/jcb/pages/submission-guidelines#videoSummaries.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Maxence Nachury, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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We have modified the alignment such that the sequences whose structures overlay in Fig. 6B are aligned in Fig. 6C.
We also modified the text lines 344-348: “The N-terminal CCDC81 domain overlays well with the structure of IJ34, notably with helices α1, α2, α7, α8, α10 and strands β1, β2, β3, β5, β6, β7 of IJ34, suggesting that it is also a microtubule-binding protein (Fig. 6B). Although the predicted structure of CCDC81 is similar to IJ34, sequence alignment reveals that their sequences are not highly conserved with the exception of β1 (Fig. 6C).”

(ii) Figure 6B should clearly show the structural similarity between IJ34 and CCDC81, given that this panel was moved from a supplementary figure to a main figure.
Currently, the two structures overlap, making it difficult to compare them. The reviewer suggests that the IJ34 and CCDC81 structures are shown separately.
We have modified Fig. 6B and now show both models overlaid and annotated according to the alignment in Fig. 6C.

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I would like to suggest not to try to void an open acknowledgement that there have been published structural studies of motile cilia tips by cryo-electron tomography in addition to negatively staining electron microscopy.

We deleted "However, this technique can sometimes introduce artifacts." and added “Ciliary tips of motile cilia from *Tetrahymena*, *Chlamydomonas*, *Trypanosoma* and mammalian sperm cells were then studied by cryo-ET (Höög et al., 2014; Reynolds et al., 2018; Zabeo et al., 2018; Zabeo et al., 2019).” (Lines 386-388).

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