CFAP61 is required for sperm flagellum formation and male fertility in human and mouse

Siyu Liu, Jintao Zhang, Zine Eddine Kherraf, Shuya Sun, Xin Zhang, Caroline Cazin, Charles Coutton, Raoudha Zouari, Shuqin Zhao, Fan Hu, Selima Fourati Ben Mustapha, Christophe Arnoult, Pierre F. Ray and Mingxi Liu
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Original submission

First decision letter

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MS TITLE: CFAP61 is required for sperm flagellum formation and male fertility in human and mouse.

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I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. From my point of view, it is particularly important to differentiate this manuscript from the paper recently published in Science bulletin by other group to warrant the novelty of this study. Furthermore, I myself came across a concern whether the mutation that you found in a human patient really cause the expression of aberrant CFAP61. You may express mutant full-length cDNA in HEK293T cells or so and check the protein expression. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make 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 attend to all of the reviewers' comments and 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. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors of this manuscript identifies CFAP61 as a disease gene linked to male infertility in a human patient, and describes a specific role for CFAP61 as a component of axonemal radial spokes in formation and function of sperm flagella in the mouse. Furthermore, the authors show that CFAP61 interacts with radial spoke components, including head and stalk regions, as well as with intraflagellar transport proteins. Overall the quality of the data is high and revealed the function of CFAP61 in mammals. The study underscores the physiological importance of CFAP61 in male fertility and will be of interest to cell and structural biologist studying flagella and motile cilia function, as well as to clinicians involved in fertility genetics. This study revealing the precise mechanism by which CFAP61 regulates sperm flagella formation and function and for further analysis human patient data.

Comments for the author

1. Genes' name for human and mouse should be italic letter.(page2 line6/line9;Figure 2A;page3 line 17;page5 line15;page15 line24;page16 line6/line10;page22 line4;page23 line14 etc.)
2."Chlamydomonas" should be in italics throughout the manuscript.
3. A period is missing in line 6 on page 5.
4. Genes' name for Chlamydomonas should be in capital italics letters.(page5 line14;page16 line12/line15/line17/line18).
5. Page5 line12:”are” should be “is”.
6. The font of "mass spectrometry" on line 2 of page 19 is different from that of other words in the manuscript.
7. Protein name for Chlamydomonas should be in capital letters(page19 line16).
8.The primer names(WDR35;IFT22;IFT74;IFT81) in Supplementary Table 2 should be lowercase.

Reviewer 2

Advance summary and potential significance to field

The paper by Lin et al., entitled ‘CFAP61 is required for sperm flagellum formation and male fertility in human and mouse’ is an interesting study on the functions of cilia-and flagella-associated protein (CFAP) 61 in sperm flagellum formation and male fertility.
Several genes are involved in the multiple morphological abnormalities of the flagella MMAF, in human. CFAP61, considered to be one of the CRC components, is an evolutionarily conserved molecule present from Chlamydomonas to humans. The authors identified an MMAF patient who has a mutation in the cfap61 gene by exome sequencing.

To prove that the cfap61 gene loss causes an MMAF-like phenotype, they produced the CFAP61-knockout male mice. They demonstrated that the cfap61 gene deletion caused abnormal sperm flagellum axoneme assembly and sperm deformity. They also performed IP-mass spectrometry of CFAP61 and revealed possible association of various molecules related to the Axoneme component, Axoneme functional regulators and Axoneme assembly.

In the meantime, a similar study was published last year: the identification of multiple MMAF patients who carry mutations at the MMAF locus and the analyses of CFAP61-deficient mice (Huang et al., 2020, Science Bulletin). It is essential to consider the overlap and differences between the two studies.

Comments for the author

1)Quality 1a) Experiments: The quality of immunostaining, IP/western blotting experiments, and the electron microscopic analyses is very nice and convincing. Overall, the experiments were adequately conducted, and the data are clearly presented.
1b) Completeness:
The fact that only one patient with CFAP61 gene abnormality has been found is insufficient for a cohort study. However, I understand that CFAP61 is the gene responsible for MMAF based on the experiments in this study and the results published by other researchers. On the other hand, the validation experiments of the candidate gene well illustrate that CFAP61 is one of the causative genes for MMAF. Although mass spectrometry is a characteristic experiment of this study, the results, unfortunately, are descriptive, and they failed to gain new insights and further directions. This interaction between CFAP61 and components of RS or CSC could be confirmed by the proximity ligation assay in the sperm context.

2) Impact:
2a) Novelty:
In Chlamydomonas, FAP61/CaM-IP3, a homolog protein of CFAP61, also known as FAP61 and CaM-IP3, were identified as one component of CSC in Chlamydomonas. Therefore CFAP61 was, to some extent, predicted to be important for the proper assembly and function of the radial spokes and the ciliary waveform. Furthermore, the sperm analyses and the histological/electron microscopy analyses of the patient study are redundant of the work by Huang et al. Therefore, it is hard to say that the topic of this work is of potential impact to this field.
b) broad interest The new findings of this study are limited to the analyses of the interacting partner molecules. I assume that this is an important result for the specific field but not for embryology in general.
Were there any new or unpredicted interacting molecules identified by the mass spectrometry analysis? If there are such molecules, and if the authors generate CRISPR-Cas9 KO mice of such gene(s) and see some effects on the structure and function of cilia this work would be worthy of publication in Development.
Otherwise, I would suggest that authors submit this paper to a more suitable journal.

First revision

Author response to reviewers' comments

Our point-by-point responses to each comment are as follows:

Reviewer 1:

Comments for the Author:

1. Genes' name for human and mouse should be italic letter.(page2 line6/line9;Figure 2A;page3 line 17;page5 line15;page15 line24;page16 line6/line10;page22 line4;page23 line14 etc.)

Response: Thank you to point out this error. We have standardized the writing of gene names for human and mouse (page2 line38/line41; Figure 2A; page2 line67; page4 line125; page9 line294; page10 line331).

2."Chlamydomonas" should be in italics throughout the manuscript.

Response: Thank you to point out this error. We have corrected the font of "Chlamydomonas" throughout the manuscript.

3. A period is missing in line 6 on page 5.

Response: Thank you to point out this error. The period has been added (page4, line116).

4. Genes' name for Chlamydomonas should be in capital italics letters.(page5 line14;page16 line12/line15/line17/line18).

Response: Thank you to point out this error. We have standardized the writing of gene names for Chlamydomonas(page4 line124; page5 line 141,142,145,147).

5. Page5 line12: "are" should be "is".

Response: Thank you to point out this error.This grammatical error has been corrected (page4, line123).

6. The font of "mass spectrometry" on line 2 of page 19 is different from that of other words in the manuscript.

Response: We have corrected the font of "mass spectrometry"(page6, line180).

7. Protein name for Chlamydomonas should be in capital letters(page19 line16).

Response: Thank you to point out this error. We have standardized the writing of protein names for Chlamydomonas(page6, line198).

8. The primer names(WDR35;IFT22;IFT74;IFT81) in Supplementary Table 2 should be lowercase.

Response: Thank you to point out this error. We have corrected the primer names to lowercase(Table S2 line21-28).

Reviewer 2 :

Comments for the Author:

1)Quality
1a) Experiments: The quality of immunostaining, IP/western blotting experiments, and the electron microscopic analyses is very nice and convincing. Overall, the experiments were adequately conducted, and the data are clearly presented.
1b) Completeness:
The fact that only one patient with CFAP61 gene abnormality has been found is insufficient for a cohort study. However, I understand that CFAP61 is the gene responsible for MMAF based on the experiments in this study and the results published by other researchers. On the other hand, the validation experiments of the candidate gene well illustrate that CFAP61 is one of the causative genes for MMAF. Although mass spectrometry is a characteristic experiment of this study, the results, unfortunately, are descriptive, and they failed to gain new insights and further directions. This interaction between CFAP61 and components of RS or CSC could be confirmed by the proximity ligation assay in the sperm context.

Response: Thank you for your appreciation and suggestions. CFAP61 was considered as a CSC rather than an RS component in previous reports. We supplemented the proximity ligation assay (PLA) to confirm that RSPH9 and CFAP61 are spatially within 40nm (Fig. 3N and page6, line 194-197), and further supplemented the evidence that CFAP61 functions as an RS component. Meanwhile, PLA also confirmed that CFAP61 interacted with serine/threonine protein kinase CSNK2A2, serine/threonine phosphotase catalytic subunit PPP1CC (Fig. S1C and page7, line 204-208), although the significance of the interaction is still unknown. It is worth noting that the deletion of CSNK2A2 or PPP1CC can cause sperm abnormalities. We supplemented the Co-IP experiment, confirming the interaction between CFAP61 and IFT81 in vivo (Fig. S7D and page9, line281-282). Interestingly, the absence of CFAP61 leads to the residue of IFT components in sperm flagella. This IFT disorder may aggravate the abnormality of sperm deformation process.
2) Impact:

2a) Novelty:
In Chlamydomonas, FAP61/CaM-IP3, a homolog protein of CFAP61, also known as FAP61 and CaM-IP3, were identified as one component of CSC in Chlamydomonas. Therefore,CFAP61 was, to some extent, predicted to be important for the proper assembly and function of the radial spokes and the ciliary waveform. Furthermore, the sperm analyses and the histological/electron microscopy analyses of the patient study are redundant of the work by Huang et al. Therefore, it is hard to say that the topic of this work is of potential impact to this field.
b) broad interest

The new findings of this study are limited to the analyses of the interacting partner molecules. I assume that this is an important result for the specific field but not for embryology in general.

Response: Thank you for your suggestions. It needs to be clarified that there is no data or mutation information from any patient in in the work of Huang et al. In addition, there is a lack of research on the axoneme of sperm cells during spermatogenesis caused by the deletion of *Cfap61*. The work by Huang et al. mainly focused on sperm deformities and abnormal motility, as well as acrosome changes during spermatogenesis, and did not provide any evidence on whether CFAP61 is an RS or CSC component. There are significant differences and progress between our work and theirs. We first reported the CFAP61 mutation carried by MMAF patients, analyzed the CFAP61 protein action network, and confirmed that CFAP61 was a component of RS and CSC. Furthermore, we also revealed a series of changes in sperm flagellum axonem after *Cfap61* deletion. Moreover, *Development* currently receives papers from outside the field of embryology.

Were there any new or unpredicted interacting molecules identified by the mass spectrometry analysis? If there are such molecules, and if the authors generate CRISPR-Cas9 KO mice of such gene(s) and see some effects on the structure and function of cilia, this work would be worthy of publication in Development.
Otherwise, I would suggest that authors submit this paper to a more suitable journal.

Response: Thank you for your suggestions. There are indeed some unpredicted CFAP61 interacting proteins by the mass spectrometry analysis. Although we cannot generate new CRISPR-Cas9 KO mice of such genes in the short term, some previous studies have shown that the function of *Cfap61* may be related to these genes. For example, *Ppp1cc* knockout can cause sperm tail deformity and dyskinesia(Sinha et al., 2013, Biology of reproduction), while *Csnk2a2* knockout mice can also cause sperm deformity(Xu et al., 1999, Nature genetics). We confirmed that these two proteins could interact with CFAP61 by proximity ligation assay (Fig. S1C and page7, line 204-208). We believe that our supplementary experiments improve the overall quality of the paper and is suitable for publication in *Development*.

Second decision letter

MS ID#: DEVELOP/2021/199805

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ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors proved Cfap61 has a unique function in the process of flagellum formation and provides a theoretical basis for differential regulation of cilia/flagellum formation and MMAF physiopathology.

Comments for the author

All my comments have been addressed in the revised manuscript and I recommend its publication.

Reviewer 2

Advance summary and potential significance to field

The authors made many efforts to make their work solid. Notably, the PLA assays show a possible interaction between CFAP61 and RSPH9 as well as CSNK2A2 and Ppp1cc. They also performed the Co-IP experiments of IFT81 to confirm the interaction.

Comments for the author

The authors have addressed my previous comments regarding the manuscript, providing an extended of the context of their findings and clarifying some of the experimental design and analysis.