



CCDC38 is required for sperm flagellum biogenesis and male fertility in mice

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AUTHORS: Ruidan Zhang, Wei Li, Li Yuan, Fei Gao, Bingbing Wu, Chao Liu, Xiuge Wang, Liying Wang, Sai Xiao, Yinghong Chen, and Huafang Wei

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 also raise ample significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. 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*

Zhang et al describe the establishment and analysis of a murine model lacking CCDC38. Loss of this gene results in male infertility, multiple defects occur during spermiogenesis. The authors claim, that the infertility is based due to CCDC38 requirement for flagellum biogenesis. While the study per se is interesting, and demonstrates the consequences of loss of this gene, several points need to be addressed, before an acceptance to Development can be recommended.

Comments for the author

1. In general, the language of the manuscript needs to be revised substantially. The reviewer recommends the help of a native scientific person to revise the manuscript. Further, the material and methods part needs to be extended to describe all methods, buffers, fixatives etc. used.
2. The IHC pictures are of substandard quality, the need to improved/replaced in order to substantiate the claims made in the text.
3. While the authors mention (line 109) Lin et al, as a reference, they do not state, that Lin published in their figure 2 expression and CCDC38 protein in various tissues. This must be noted.
4. Further to that, Lin et al demonstrate, that the Protein is localized to spermatogonia and spermatocytes. This discrepancy to their own findings must be discussed. In this respect the reviewer would like to see an experiment demonstrating the specificity of the CCDC38 antibody used in this study.
5. The establishment of the CCDC38 deficient mice generates animals heterozygous for the mutation. No information is given at any time about the phenotype/fertility of such het males - include and comment on the results.
6. Gif 2 C - the way Primer F1 and R2 are positioned, they would generate a band in the ko situation as well since R2 position is not deleted (if the lines in 2C are correct).
7. Line 135 claims that results of female mice are shown in Fig 2 F - this is not the case.
8. The reviewer does not see an apparent difference in morphology as claimed by the authors to be visible in Figure 4A stage 9 (line 157).
9. Line 170 - the claim is not substantiated by the Figure 5A - replace with better image 10. Line 174, line 180 - the claims are not supported by Figure 5C 11. Fig 5 D - explain, why the TEM images of wt are of different size (100nm) compared to ko (100um). This makes comparison of the data difficult.
12. Figure 6A - the wt and the ko pictures seem to be taken from different angles of the sperm head making a comparison problematic.
13. Fig.6C Western Blots seem to present a composite of different experiments since the bands displayed seem not to match (IFT20 broader signal compared to IFT88).
14. Same holds true for Fig 8b, Western Blot of ODF1 and ODF2.

Reviewer 2*Advance summary and potential significance to field*

In this manuscript, Zhang et al identify a new CCDC42-interacting protein CCDC38, and demonstrate it is essential for the sperm flagellum biogenesis in mouse. They show that CCDC38 localizes on the manchette of spermatids and the sperm tail, and disruption of CCDC38 resulted in defects in sperm tail biogenesis, in turn, which caused male infertility. In addition, they find that CCDC38 interacts with IFT88 and ODF2, and propose that CCDC38 collaborates with the intraflagellar transport pathways to facilitates ODF2 transportation in flagella. While this is a potentially interesting observation, there are some concerns about some data as detailed below.

Comments for the author

In this manuscript, Zhang et al identify a new CCDC42-interacting protein, CCDC38, and demonstrate it is essential for the sperm flagellum biogenesis in mouse. They show that CCDC38 localizes on the manchette of spermatids and the sperm tail, and disruption of CCDC38 resulted in defects in sperm tail biogenesis, in turn, which caused male

infertility. In addition, they find that CCDC38 interacts with IFT88 and ODF2, and propose that CCDC38 collaborates with the intraflagellar transport pathways to facilitate ODF2 transportation in flagella. While this is a potentially interesting observation, there are some concerns about some data as detailed below.

1. Authors' previous work showed that CFAP53 could also interact with IFT88 and CCDC42 to participate in sperm flagellum biogenesis. Whether CCDC38 complexes with CFAP53? The relationship among these four proteins should be discussed.

2. It has been reported that CCDC42 could bind to both ODF1 and ODF2 (PMID: 31475146). Although authors showed the protein level of ODF1 was normal in *Ccdc38* knockout mouse, it was very difficult to exclude ODF1 as the TEM showed the outer dense fiber was perturbed in *Ccdc38* knockout mouse. Therefore, the interaction between CCDC38 and ODF1 should be detected.

3. Many sperm flagellum biogenesis related proteins have also been reported to be required for respiratory cilia. Whether the disruption of CCDC38 may also perturb the respiratory cilia on tracheal epithelial cells?

Minor comments:

Fig 1C, loss of negative control.

Fig 1D, Step 11-12 spermatid is incorrect, which may be Step 9-10. Similar problems could also be found in Fig 6A, Fig 7E.

Fig 2E, 7A-C, 8A,B The mass rulers for Western blots are always missing.

Lane 134 "in contrast, female *Ccdc38*^{-/-} mice generated offspring after mating with WT adult males (Fig. 2F)" Fig 2F does not show female *Ccdc38*^{-/-} mice fertility.

Fig 2H "the mean \pm SD" is not same with the description in "Statistical Analysis" part.

Fig 4B shows same photo in Step 6 and Step 7.

Fig 5A, lane 546 "Arrows indicated the abnormal sperm", is it correct? Furthermore, does the arrow indicate the same defect in control mice? Similar problems are shown in Fig 5B, 5C, 6B.

Fig 5D, the ultrastructure of *Ccdc38*^{-/-} sperm flagellum is not clear, and the bar is problematic. The diameter of axoneme in *Ccdc38*^{-/-} sperm end piece is almost 1 μ m!

Fig 8E lane 593 "white arrows indicated the discontinuous, punctiform short, white arrowhead

indicated the tenuous axoneme" These labels are incorrect.

There are many language errors in the text that need careful correcting the spelling, grammar, word use throughout this manuscript.

First revision

Author response to reviewers' comments

Reviewer 1

Advance Summary and Potential Significance to Field...

Zhang et al describe the establishment and analysis of a murine model lacking CCDC38. Loss of this gene results in male infertility, multiple defects occur during spermiogenesis. The authors claim, that the infertility is based due to CCDC38 requirement for flagellum biogenesis. While the study per se is interesting, and demonstrates the consequences of loss of this gene, several points need to be addressed, before an acceptance to Development can be recommended.

Reviewer 1 Comments for the Author...

1. In general, the language of the manuscript needs to be revised substantially. The reviewer recommends the help of a native scientific person to revise the manuscript. Further, the material and methods part needs to be extended to describe all methods, buffers, fixatives etc. used.

Response: Thank you for pointing out this problem. We have thoroughly revised the manuscript with the help of a native English speaker, and we have expanded the materials and methods section as below:

Materials and methods

Antibodies

Mouse anti-GFP antibody (M20004L, Abmart) and rabbit anti-MYC antibody (BE2011, Abmart) were each used at a dilution of 1:1000 for western blotting. ODF2 antibody (12058-1-AP, Proteintech) was used at a dilution of 1:1000 for western blotting and 1: 200 for immunofluorescence. Mouse anti- α -TUBULIN antibody (AC012, Abclonal) was used at a dilution of 1:100 for immunofluorescence. Mouse anti- α /B-TUBULIN antibody (ab44928, Abcam) was used at a dilution of 1: 100 for immunofluorescence. Mouse anti-GAPDH antibody (AC002, Abclonal) was used at a dilution of 1:10000 for western blotting. Mouse anti-ODF1 antibody (sc-390152, Santa Cruz) was used at a dilution of 1:500 for western blotting. Mouse anti-Ac-TUBULIN antibody (T7451, Sigma-Aldrich) was used at a dilution of 1:200 for immunofluorescence. Mouse anti- CCDC38 were generated by Dia-an Biotech (Wuhan, China) and was used at a dilution of 1:20 for immunofluorescence. Rabbit anti-CCDC38 were generated by Dia-an Biotech (Wuhan, China) and was used at a dilution of 1:500 for western blotting. The Alexa Fluor 488 conjugate of lectin PNA (1:400, L21409, Thermo Fisher) was used for immunofluorescence. Secondary antibodies were goat anti-rabbit FITC (1:200, ZF- 0311, Zhong Shan Jin Qiao), goat anti-TRITC (1:200, ZF-0316, Zhong Shan Jin Qiao), goat anti-mouse FITC (1:200, ZF-0312, Zhong Shan Jin Qiao), and goat anti-rabbit TRITC (1:200, ZF0313, Zhong Shan Jin Qiao).

Immunoprecipitation

Transfected cells were lysed in a lysis buffer (50 mM HEPES, PH 7.4, 250 mM NaCl, 0.1% NP-40 containing PIC and PMSF) on ice for 30 min, and centrifugated at 12000 rpm at 4°C for 15 min, cell lysates were incubated with primary antibody overnight at 4°C, next incubated with protein A-Sepharose (GE, 17-1279-03) for 2 h at 4°C, then the precipitants were washed 3 times with lysis buffer, then added the SDS loading buffer for 10 min at 95°C and subjected to immunoblotting analysis.

Transmission electron microscopy

The methods were as reported previously with some modifications (Liu et al., 2016). The testis from WT and *Ccdc38* depletion mice testis and epididymis were dissected and fixed in 2.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer at 4°C overnight. After washing in 0.1 M cacodylate buffer, samples were cut into small pieces, then immersed in 1% OsO₄ for 1h at 4°C. Samples were dehydrated through a graded acetone series (50%, 60%, 70%, 80%, 90%, 95%, 100%) and embedded in resin (DDSA, NMA, enhancer, 812) for staining. Ultrathin sections were cut and stained with uranyl acetate and lead citrate, images were acquired and analyzed using a JEM-1400 transmission electron microscope.

Statistical Analysis

All data are presented as the $\text{mean} \pm \text{SD}$. Statistical significance of the differences between the mean values for the various genotypes was measured by Student's t-tests with paired, 2-tailed distribution. The data were considered significant when *P*-values were less than 0.05(*), 0.01(**) or 0.001(***)

LIU, C., WANG, H., SHANG, Y., LIU, W., SONG, Z., ZHAO, H., WANG, L., JIA, P., GAO, F., XU, Z., et al. (2016). Autophagy is required for ectoplasmic specialization assembly in sertoli cells. *Autophagy*, 12, 814-32.

2. The IHC pictures are of substandard quality, the need to improved/replaced in order to substantiate the claims made in the text.

Response: Thank you for your suggestion. We have replaced some of our IHC pictures with higher resolution.

3. While the authors mention (line 109) Lin et al, as a reference, they do not state, that Lin published in their figure 2 expression and CCDC38 protein in various tissues. This must be noted.

Response: Thank you for your suggestion. *Lin et al.* have reported that *Ccdc38* is a testes-specific gene and predominantly expressed in the testis in Figure 2 (Lin et al., 2016). We have corrected the sentences as guided by *Lin et al.*'s work. (please see line 116-117):

CCDC38, exclusively expressed in testes as reported.

LIN, S. R., LI, Y. C., LUO, M. L., GUO, H., WANG, T. T., CHEN, J. B., MA, Q., GU, Y. L., JIANG, Z. M. and GUI, Y. T. (2016). Identification and characteristics of the testes-specific gene, *Ccdc38*, in mice. *Mol Med Rep*, 14, 1290-6.

4. Further to that, Lin et al demonstrate, that the Protein is localized to spermatogonia and spermatocytes. This discrepancy to their own findings must be discussed. In this respect the reviewer would like to see an experiment demonstrating the specificity of the CCDC38 antibody used in this study.

Response: Thank you for your suggestion. Our antibody to CCDC38 has been validated with both western blot and immunofluorescence. We have discussed the discrepancy between their and our results (please see line 248-256):

Ccdc38 is exclusively expressed in testes (Lin et al., 2016), but its role during spermiogenesis has not yet been investigated. To study its role during spermiogenesis, we generated a *Ccdc38*^{-/-} mouse model and found the *Ccdc38*^{-/-} male mice to be sterile (Fig. 2F) due to significantly reduced spermatozoa number and motility (Fig. 3B, C). As for which kinds of cells were affected by CCDC38, a discrepancy exists between our results and others. *Lin et al.* previously reported that CCDC38 is mainly localized to spermatogonia and spermatocytes (Lin et al., 2016), but we found that CCDC38 mainly participates in spermatid elongation and is localized on the manchette and sperm tail (Fig. 1D, 6C). The discrepancy may result from different CCDC38 antibodies being used. Our CCDC38 antibody had been validated by both western blotting (Fig. 2E) and immunofluorescence (Fig. 6C), supporting the robustness of our antibody labeling and the findings of our protein localization in this study.

LIN, S. R., LI, Y. C., LUO, M. L., GUO, H., WANG, T. T., CHEN, J. B., MA, Q., GU, Y. L., JIANG, Z. M. and GUI, Y. T. (2016). Identification and characteristics of the testes-specific gene, *Ccdc38*, in mice. *Mol Med Rep*, 14, 1290-6.

5. The establishment of the CCDC38 deficient mice generates animals heterozygous for the mutation. No information is given at any time about the phenotype/fertility of such het males - include and comment on the results.

Response: Thank you for your suggestion! We didn't find any defects in the heterozygous male mice. We have added the phenotype and fertility of heterozygous male mice in Figure 2F-K and Figure 3A-D.

6. Gif 2 C - the way Primer F1 and R2 are positioned, they would generate a band in the ko situation as well, since R2 position is not deleted (if the lines in 2C are correct).

Response: Thank you for pointing out this problem. We have corrected the position of primer R2 in Figure 2C as below.

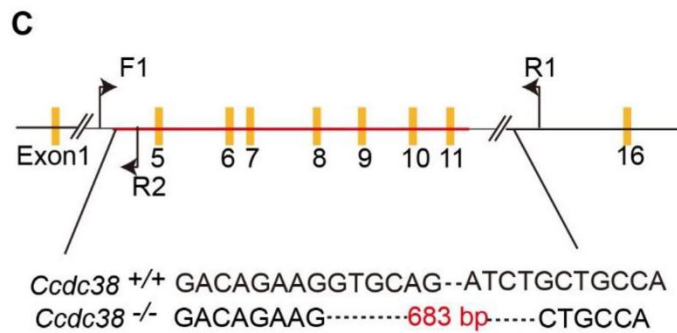


Fig. 2. *Ccdc38* knockout leads to male infertility.

(C) The generation of *Ccdc38*^{-/-} mice lacking exon 5-11.

7. Line 135 claims that results of female mice are shown in Fig 2 F - this is not the case.

Response: Thank you for pointing out this problem. We have revised this information as below (please see line 144-146):

We then examined the fertility of *Ccdc38*^{-/-} mice. Male *Ccdc38*^{-/-} mice exhibited normal mounting behaviors and produced coital plugs but failed to produce any offspring after mating with WT adult female mice (Fig. 2F), in contrast, female *Ccdc38*^{-/-} mice could generate offspring after mating with WT adult males, which was similar to that of the *Ccdc38*^{+/+} female mice (Fig. 2G).

8. The reviewer does not see an apparent difference in morphology as claimed by the authors to be visible in Figure 4A stage 9 (line 157).

Response: Thank you for pointing out this problem. We have revised our manuscripts as shown below (please see line 170-172):

In *Ccdc38*^{+/+} mice testis sections, round spermatids differentiated into elongating spermatids from stage IX, while abnormal elongated spermatids occurred at stage X in *Ccdc38*^{-/-} mice testis (Fig. 4A).

9. Line 170 - the claim is not substantiated by the Figure 5A - replace with better image

Response: Thank you for pointing out this problem! We have corrected Figure 5A as below:

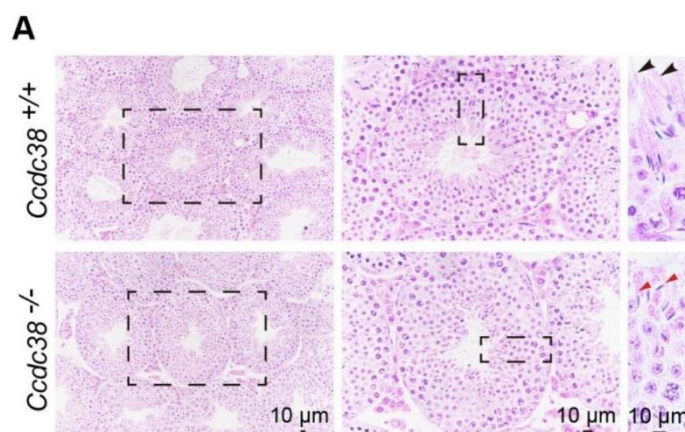


Fig. 5. The flagellum is disorganized in *Ccdc38*^{-/-} spermatids.

(A) The histology of the seminiferous tubules from *Ccdc38*^{+/+} and *Ccdc38*^{-/-} male mice. Black arrowheads indicate normal sperm tails in the *Ccdc38*^{+/+} mice testis seminiferous tubule, red arrowheads indicate the abnormal sperm flagellum in the *Ccdc38*^{-/-} mice testis seminiferous tubule.

10. Line 174, line 180 - the claims are not supported by Figure 5C

Response: Thank you for pointing out this problem. We have modified the immunofluorescence staining with the sperm flagellum marker Ac-TUBULIN in the new Figure 5C, as below :

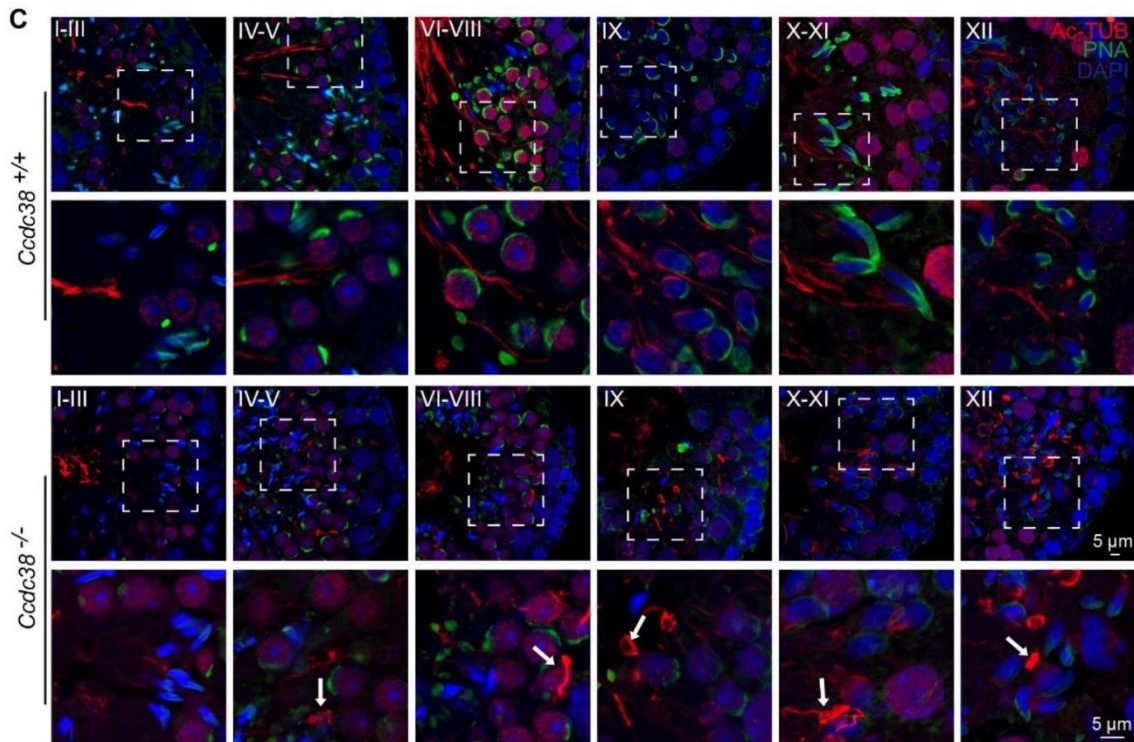


Fig. 5. The flagellum is disorganized in *Ccdc38*^{-/-} spermatids.

(C) Immunofluorescence analysis of Ac-TUBULIN (red) and PNA lectin (green) to identify sperm flagellum biogenesis. White arrows indicate short tails were found from stage IV-V in *Ccdc38*^{-/-} mice as compared with the control group.

11. Fig 5 D - explain, why the TEM images of wt are of different size (100nm) compared to ko (100um). This makes comparison of the data difficult.

Response: Thank you for pointing out this problem. We have replaced with new TEM images as shown below with Figure 5D:

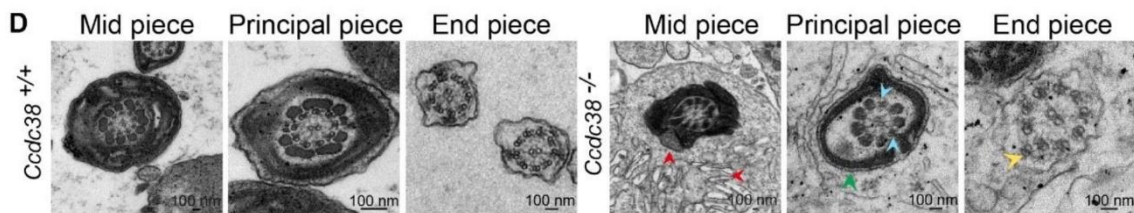


Fig. 5. The flagellum is disorganized in *Ccdc38*^{-/-} spermatids.

(D) Cross-sections of *Ccdc38*^{-/-} sperm tails revealed the disorganization of axonemal microtubules and tail accessory structures. Red arrowheads indicate abnormal mitochondrial and the cytoplasm, blue arrowheads indicate the loss of outer dense fibers, the green arrowhead indicates the abnormal fibrous sheath, and the yellow arrowhead indicates the abnormal axoneme. Scale bars: 10 μm (A, B); 5 μm (C); 100 nm (D).

12. Figure 6A - the wt and the ko pictures seem to be taken from different angles of the sperm head making a comparison problematic.

Response: Thank you for your suggestion. We have revised the images as below:

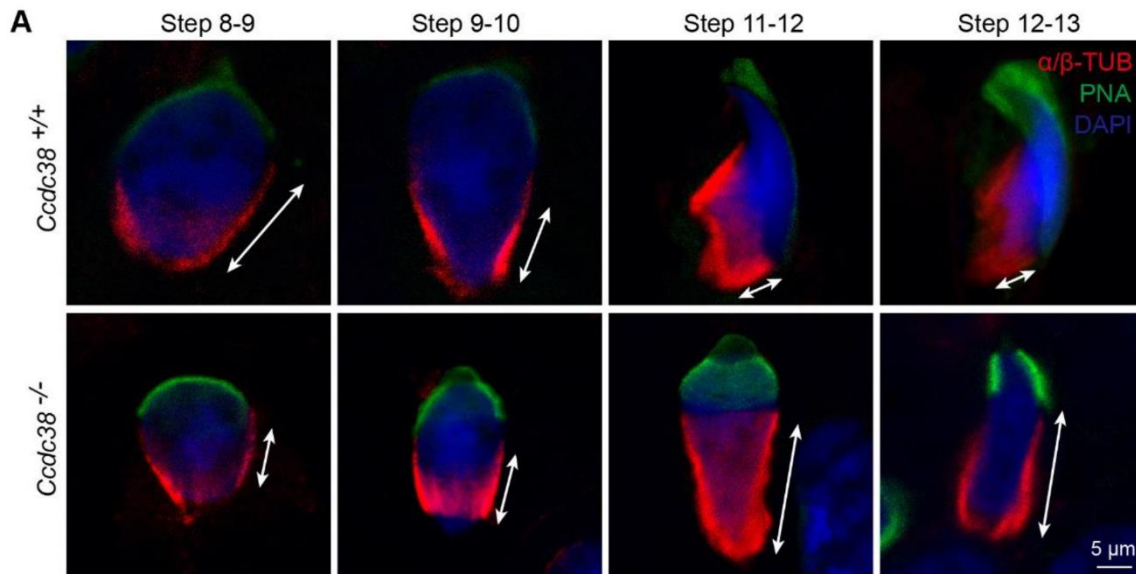
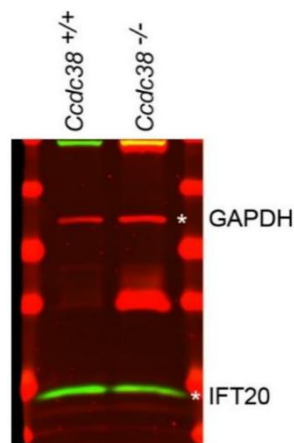


Fig. 6. The manchette is ectopically placed in *Ccdc38*^{-/-} spermatids.

(A) Abnormal manchette elongation in *Ccdc38*^{-/-} spermatids. Spermatids from different manchette-containing steps were stained with α/β -TUBULIN antibody (red) and PNA lectin (green, acrosome marker) to visualize the manchette. *Ccdc38*^{-/-} spermatids displayed abnormal elongation of the manchette.

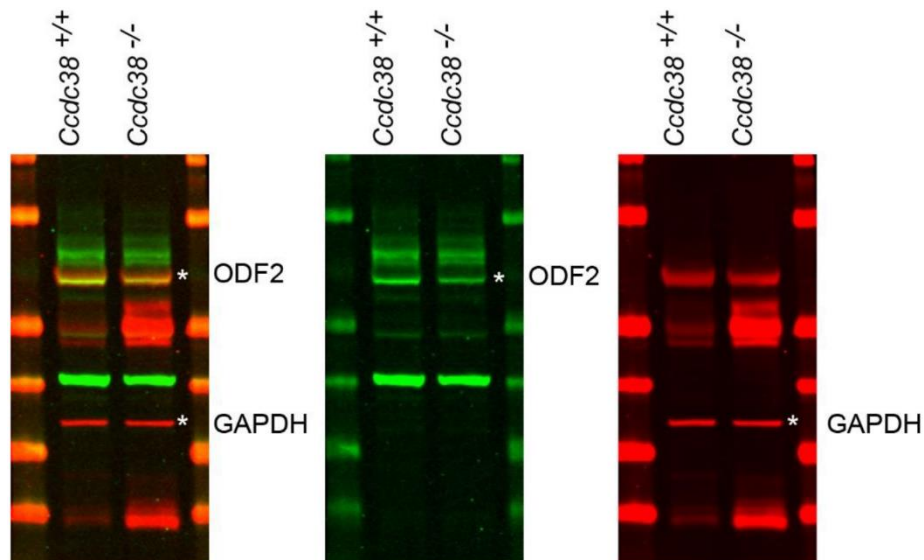
13. Fig.7C Western Blots seem to present a composite of different experiments since the bands displayed seem not to match (IFT20 broader signal compared to IFT88).

Response: Thank you for pointing out this problem. All western blots were generated from the same experiments. The original images of IFT20 and GAPDH are shown below:



14. Same holds true for Fig 8b, Western Blot of ODF1 and ODF2.

Response: All western blots were generated from the same experiments. The original images of ODF2 and GAPDH are provided below:



Review2

In this manuscript, Zhang et al identify a new CCDC42-interacting protein, CCDC38, and demonstrate it is essential for the sperm flagellum biogenesis in mouse. They show that CCDC38 localizes on the manchette of spermatids and the sperm tail, and disruption of CCDC38 resulted in defects in sperm tail biogenesis, in turn, which caused male infertility. In addition, they find that CCDC38 interacts with IFT88 and ODF2, and propose that CCDC38 collaborates with the intraflagellar transport pathways to facilitates ODF2 transportation in flagella. While this is a potentially interesting observation, there are some concerns about some data as detailed below.

1. Authors' previous work showed that CFAP53 could also interact with IFT88 and CCDC42 to participate in sperm flagellum biogenesis. Whether CCDC38 complexes with CFAP53? The relationship among these four proteins should be discussed.

Response: Thank you for pointing out this problem. We have now tested this relationship, and found that CCDC38 does indeed interact with CFAP53. The findings are shown in Figure 7B in the new version of this manuscript.

The relationship between these four proteins have been discussed as below (please see line 293-299):

Previously, we found that CFAP53 could interact with both CCDC42 and IFT88 to regulate the anterograde transport along the flagellum (Wu et al., 2021). In this study, we found that CCDC38 also interacts with CFAP53 (Fig. 7B). Thus, CCDC38 may interact with CFAP53, CCDC42 and IFT88 to regulate cargo transport by IMT and IFT during flagellum biogenesis. Further investigations are needed to reveal the detailed roles of these proteins and their relationships during flagellum biogenesis.

WU, B., YU, X., LIU, C., WANG, L., HUANG, T., LU, G., CHEN, Z. J., LI, W. and LIU, H. (2021). Essential Role of CFAP53 in Sperm Flagellum Biogenesis. *Front Cell Dev Biol*, 9, 676910.

2. It has been reported that CCDC42 could bind to both ODF1 and ODF2 (PMID: 31475146). Although authors showed the protein level of ODF1 was normal in Ccdc38 knockout mouse, it was very difficult to exclude ODF1 as the TEM showed the outer dense fiber was perturbed in Ccdc38 knockout mouse. Therefore, the interaction between CCDC38 and ODF1 should be detected.

Response: Thank you for pointing out this problem! We have examined the relationship between CCDC38 and ODF1 (Fig. S3), and found that CCDC38 indeed could immunoprecipitate ODF1 as below:

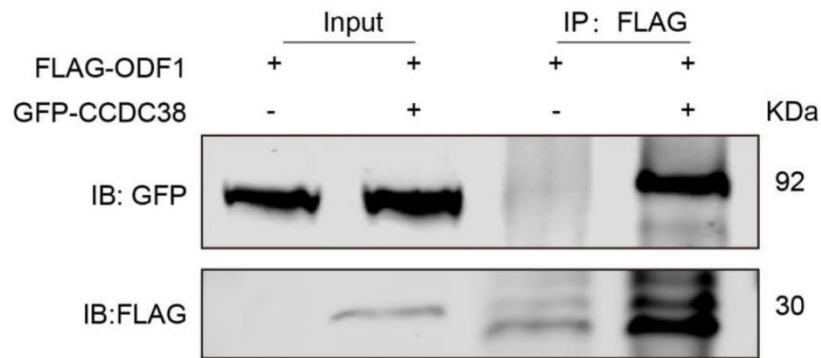


Fig. S3. CCDC38 can interact with ODF1. pRK-FLAG-ODF1 and pEGFP-C1-CCDC38 plasmids were transfected into HEK293T cells. Forty-eight hours after transfection, cells were collected for immunoprecipitation with anti-FLAG and analyzed with GFP or FLAG antibodies, respectively.

3. Many sperm flagellum biogenesis related proteins have also been reported to be required for respiratory cilia. Whether the disruption of CCDC38 may also perturb the respiratory cilia on tracheal epithelial cells?

Response: Thank you for pointing out this problem! We have examined the respiratory cilia on tracheal epithelial cells by H&E and SEM (Fig. S2), we found no obvious differences between *Ccdc38*^{+/+} and *Ccdc38*^{-/-} mice as below:

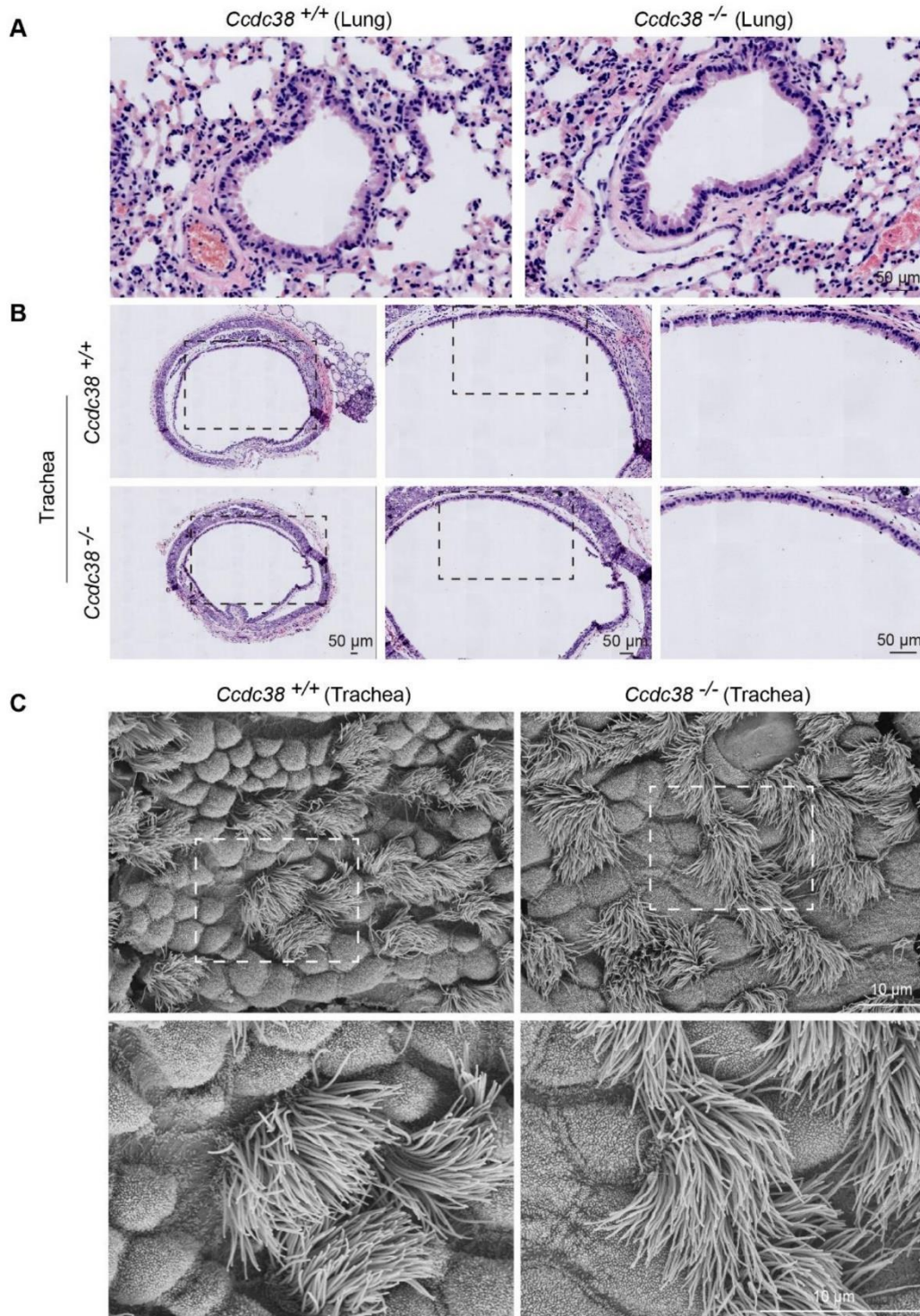


Fig. S2. Trachea cilia and lung cilia appear normal in *Ccdc38*^{-/-} mice. (A) The histology of the lung from *Ccdc38*^{+/+} and *Ccdc38*^{-/-} mice. (B) The histology of the trachea from *Ccdc38*^{+/+} and *Ccdc38*^{-/-} mice. (C) Scanning electron micrography of *Ccdc38*^{+/+} and *Ccdc38*^{-/-} tracheal epithelium at low (upper) and high magnifications of the boxed areas (lower). Scale bars: 50 μm (A, B); 10 μm (C).

Minor comments:

4. Fig 1C, loss of negative control.

Response: Thank you for your suggestion. All these methods have been well-developed in the lab; therefore, these negative controls were not presented in the original manuscript to avoid distracting the readers. We have now included negative controls in Figures S1.

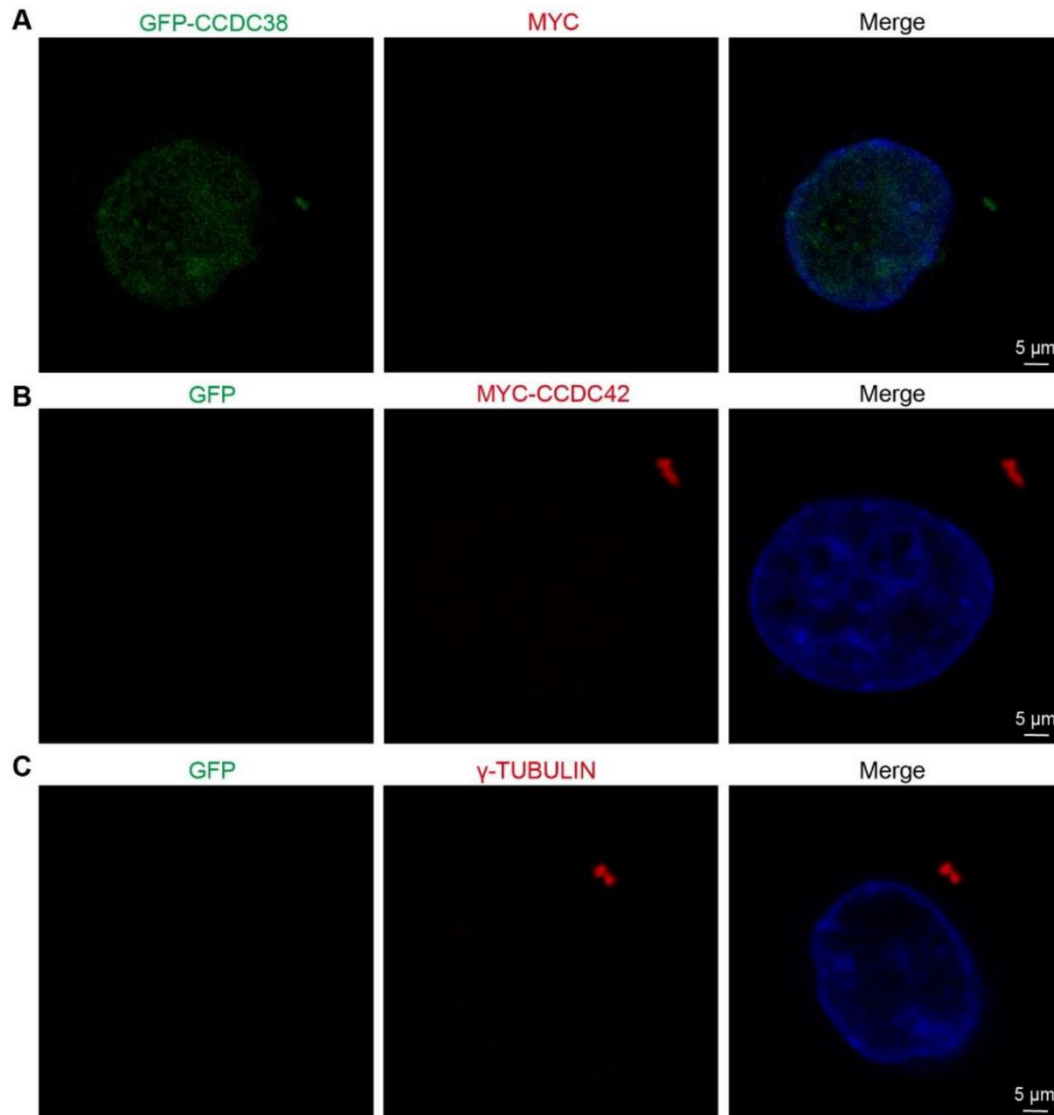


Fig. S1. Immunofluorescence analysis shows the localization of CCDC38 in somatic cells. (A-B) Immunofluorescence analysis using anti-GFP (green) and anti- MYC (red) antibodies was performed in Hela cells. Nuclei were stained with DAPI (blue). (C) Immunofluorescence analysis using anti-GFP (green) and anti- γ -TUBULIN (red) antibodies were performed in Hela cells. Nuclei were stained with DAPI (blue). Scale bars: 5 μ m.

5. Fig 1D, Step 11-12 spermatid is incorrect, which may be Step 9-10. Similar problems could also be found in Fig 6A, Fig 7E.

Response: Thank you for your suggestion. We have modified the labeling of these images as shown below:

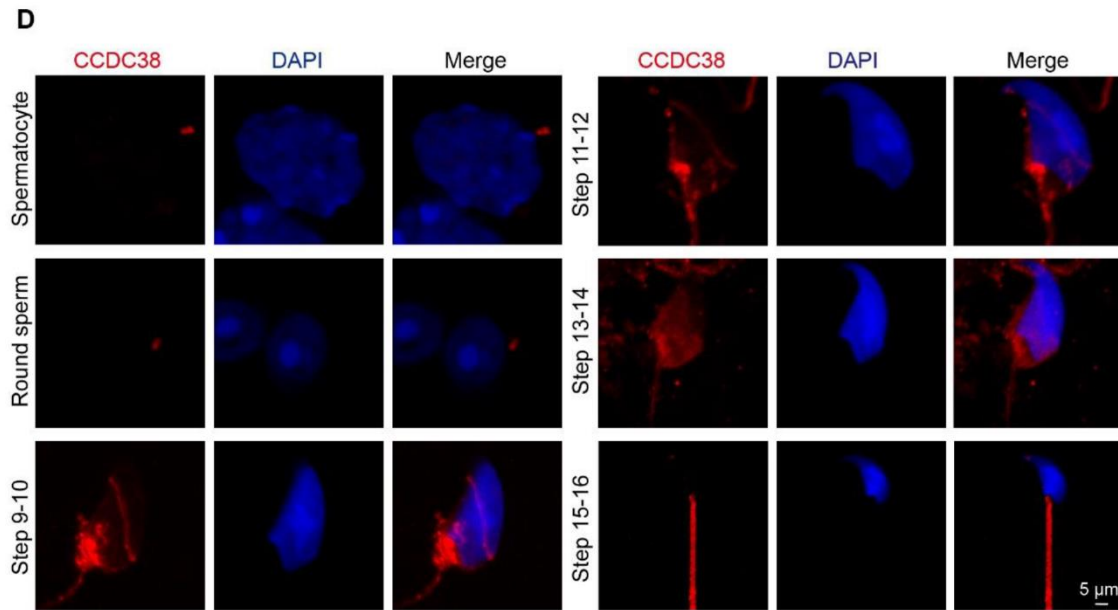


Fig. 1. CCDC38 interacts with CCDC42.

(D) Testicular germ cells were stained with anti-CCDC38 antibody, and the nuclei were stained with DAPI. Scale bars: 5 μ m.

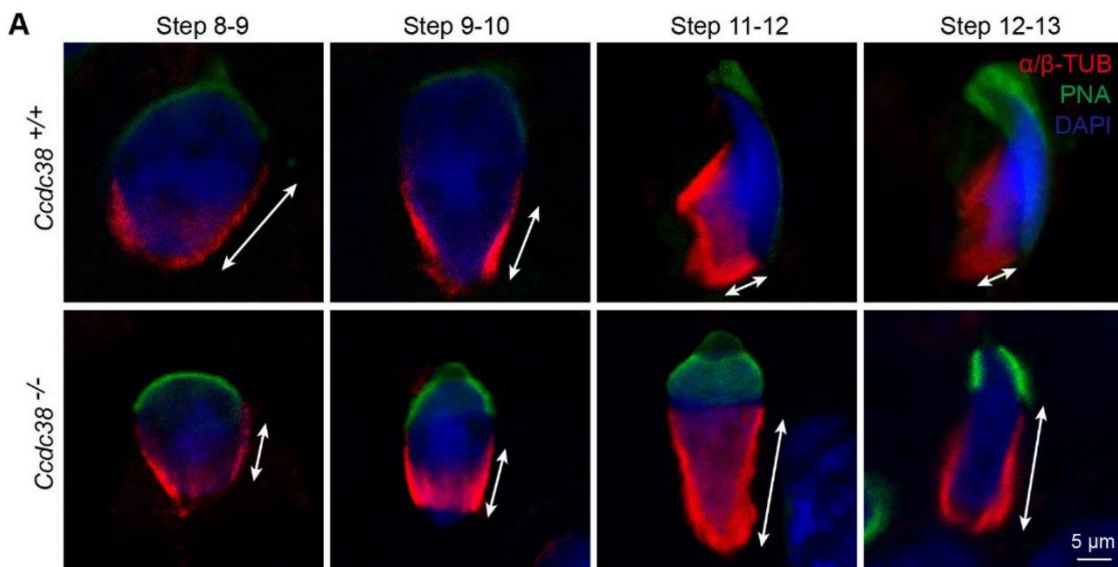


Fig. 6. The manchette is ectopically placed in *Ccdc38*^{-/-} spermatids.

(A) Abnormal manchette elongation in *Ccdc38*^{-/-} spermatids. Spermatids from different manchette-containing steps were stained with α/β -TUBULIN antibody (red) and PNA lectin (green, acrosome marker) to visualize the manchette. *Ccdc38*^{-/-} spermatids displayed abnormal elongation of the manchette.

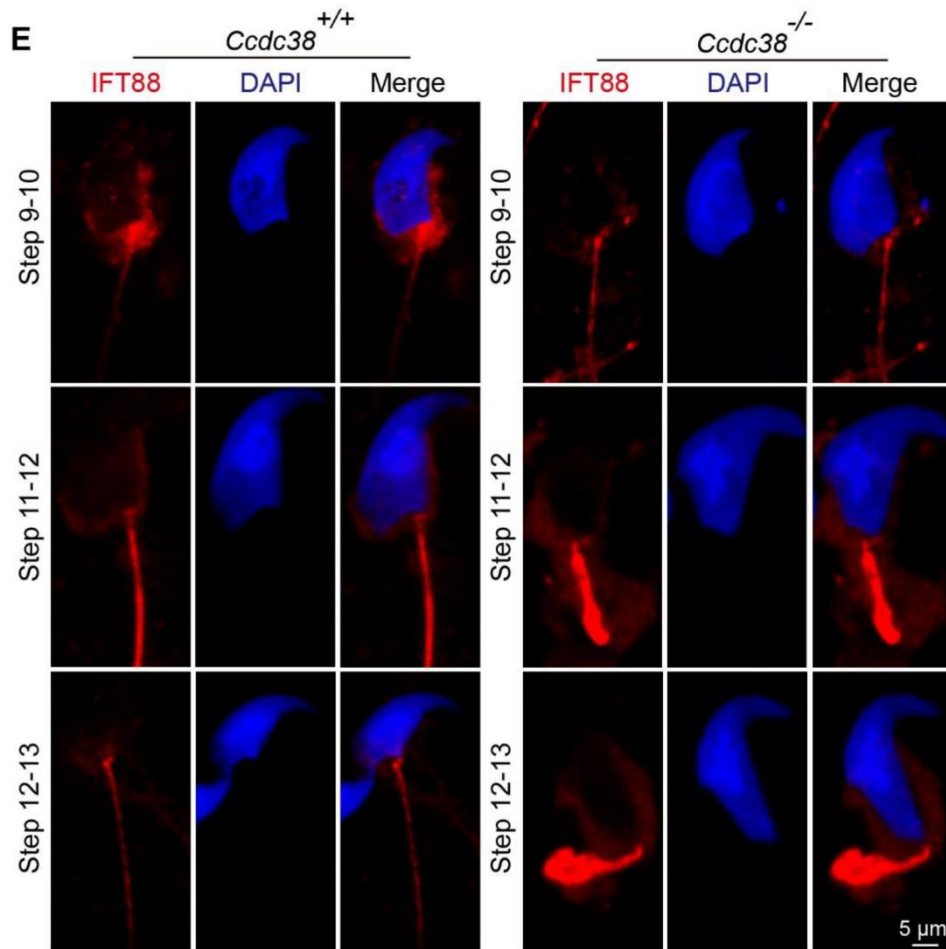


Fig. 7. CCDC38 interacts with IFT88.

(E) Immunofluorescence of IFT88 (red) and DAPI (blue) in different-stage spermatids from *Ccdc38*^{+/+} and *Ccdc38*^{-/-} mice. Data are presented as the mean \pm SD. Scale bars: 5 μ m.

6. Fig 2E, 7A-C, 8A, B The mass rulers for Western blots are always missing. Response: Thank you for pointing out this problem! We have added the mass rulers for each western blot in the revised manuscript.

7. Lane 134 “in contrast, female *Ccdc38*^{-/-} mice generated offspring after mating with WT adult males (Fig. 2F)” Fig 2F does not show female *Ccdc38*^{-/-} mice fertility.

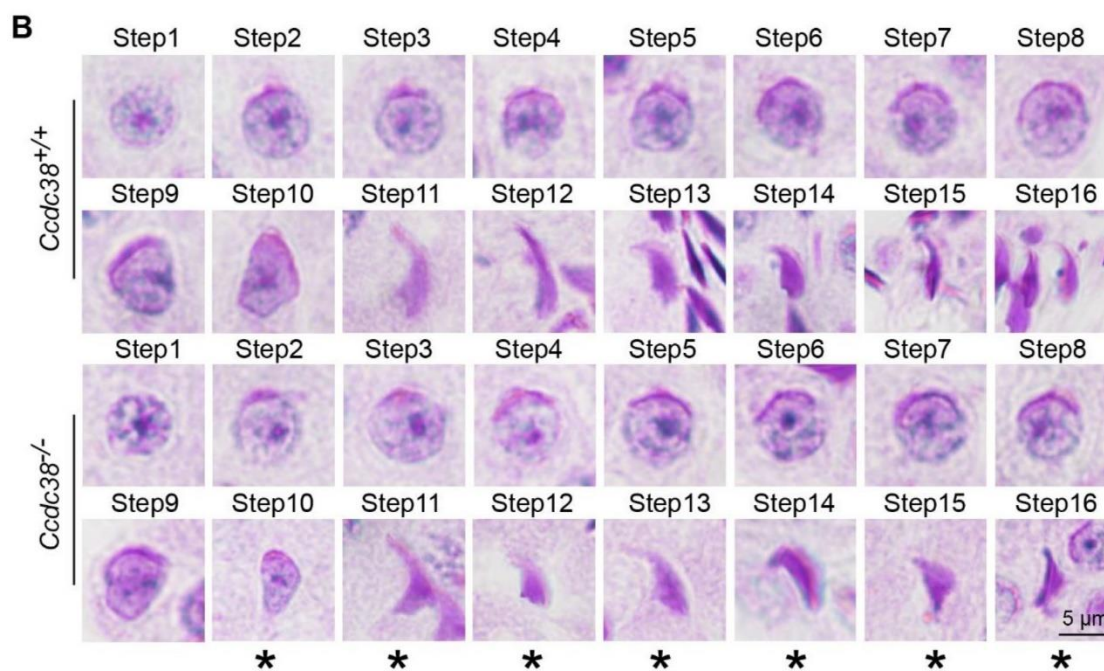
Response: Thank you for pointing out this problem! We have revised this information as shown above (please see line 144-146).

8. Fig 2H “the mean \pm SD” is not same with the description in “Statistical Analysis” part.

Response: Thank you for pointing out this problem! We have revised the statistical analysis in the revised manuscript.

9. Fig 4B shows same photo in Step 6 and Step 7.

Response: Thanks for pointing out this key problem with our manuscript! We have revised the images as below:



(B) PAS staining of spermatids at different steps from *Ccdc38*^{+/+} and *Ccdc38*^{-/-} mice. The asterisks indicate abnormal spermatid shapes found starting at step 10. Scale bars: 10 µm (A); 5 µm (B).

10. Fig 5A, lane 546 “Arrows indicated the abnormal sperm”, is it correct? Furthermore, does the arrow indicate the same defect in control mice? Similar problems are shown in Fig 5B, 5C, 6B.

Response: Thank you for pointing out this key problem with our manuscript! Arrows indicate the sperm in both control and *Ccdc38*-knockout mice. We have updated our manuscript as below:

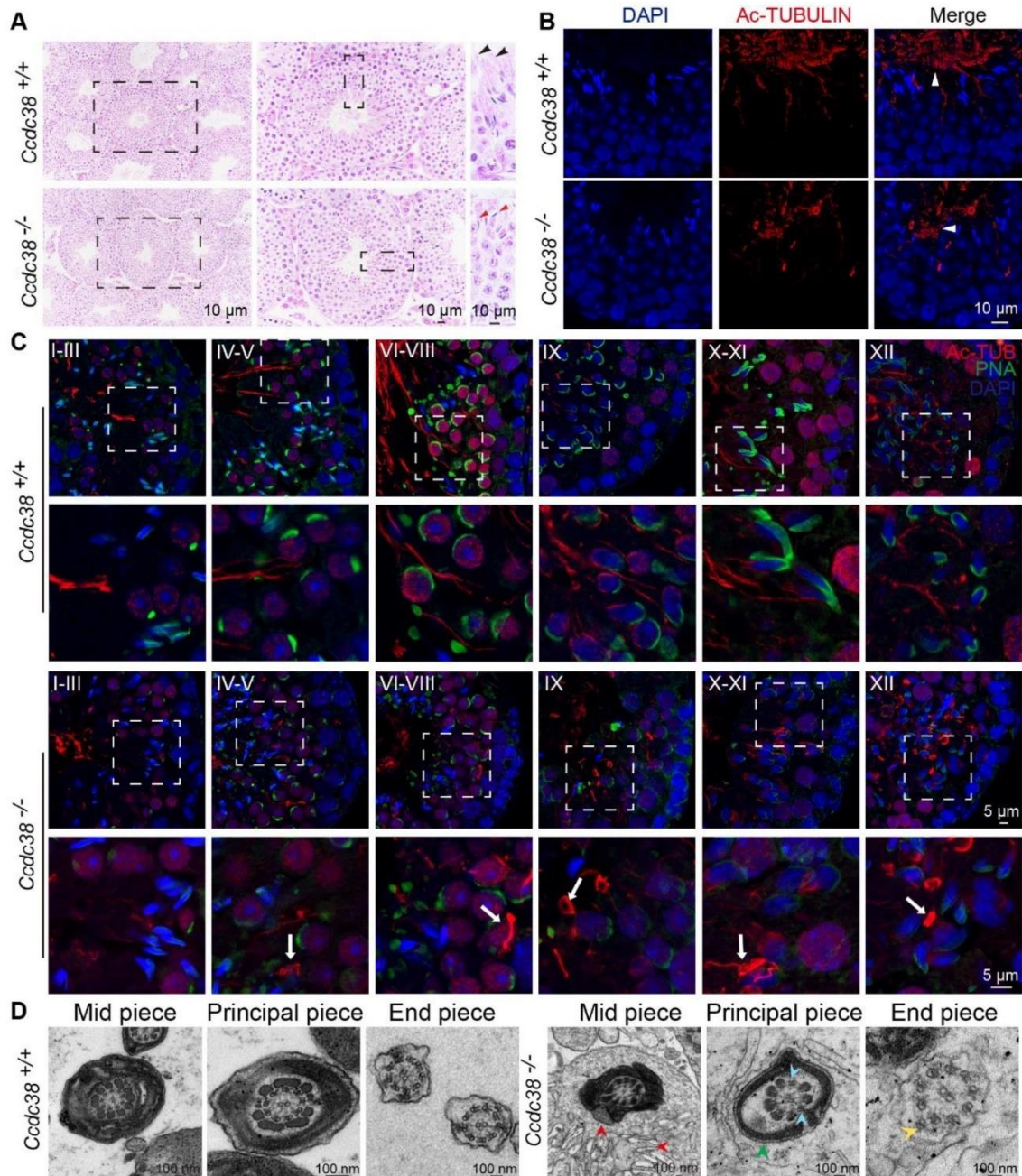


Fig. 5. The flagellum is disorganized in *Ccdc38*^{-/-} spermatids. (A) The histology of the seminiferous tubules from *Ccdc38*^{+/+} and *Ccdc38*^{-/-} male mice. Black arrowheads indicate normal sperm tails in the *Ccdc38*^{+/+} mice testis seminiferous tubule, red arrowheads indicate the abnormal sperm flagellum in the *Ccdc38*^{-/-} mice testis seminiferous tubule. (B) Immunofluorescence analysis of Ac-TUBULIN (red) antibodies in *Ccdc38*^{-/-} mice testes showed flagellar defects. The nucleus was stained with DAPI (blue) and white arrows indicate the sperm flagellum in *Ccdc38*^{+/+} and *Ccdc38*^{-/-} mice. (C) Immunofluorescence analysis of Ac-TUBULIN (red) and PNA lectin (green) to identify sperm flagellum biogenesis. White arrows indicate short tails were found from stage IV-V in *Ccdc38*^{-/-} mice as compared with the control group. (D) Cross-sections of *Ccdc38*^{-/-} sperm tails revealed the disorganization of axonemal microtubules and tail accessory structures. Red arrowheads indicate abnormal mitochondrial and the cytoplasm, blue arrowheads indicate the loss of outer dense fibers, the green arrowhead indicates the abnormal fibrous sheath, and the yellow arrowhead indicates the abnormal axoneme. Scale bars: 10 μ m (A, B); 5 μ m (C); 100 nm (D).

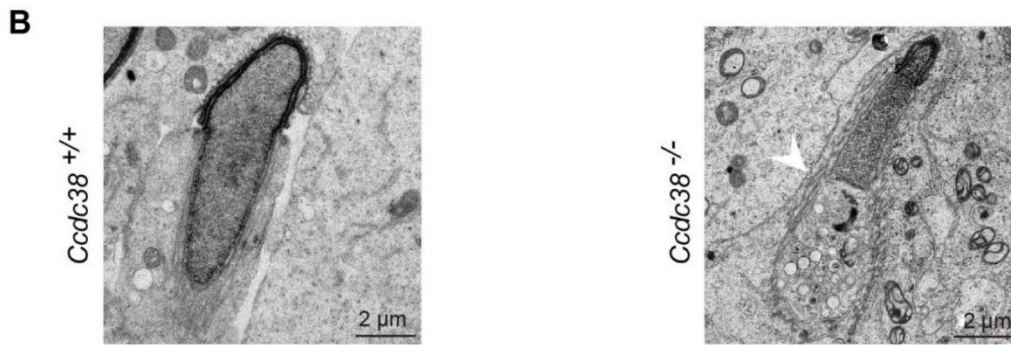


Fig. 6. The manchette is ectopically placed in *Ccdc38*^{-/-} spermatids.

(B) TEM revealed that the manchette of elongating spermatids (steps 9-11) from *Ccdc38*^{-/-} mice were ectopically placed. The white arrowhead indicates the abnormal manchette.

11. Fig 5D, the ultrastructure of *Ccdc38*^{-/-} sperm flagellum is not clear, and the bar is problematic. The diameter of axoneme in *Ccdc38*^{-/-} sperm end piece is almost 1μm!

Response: Thank you for pointing out this key problem with our manuscript! We have replaced the TEM image in Fig.6, as shown above.

12. Fig 8E lane 593 “white arrows indicated the discontinuous, punctiform short, white arrowhead indicated the tenuous axoneme” These labels are incorrect.

Response: Thank you for pointing out this problem! We have updated Figure 8E as below:

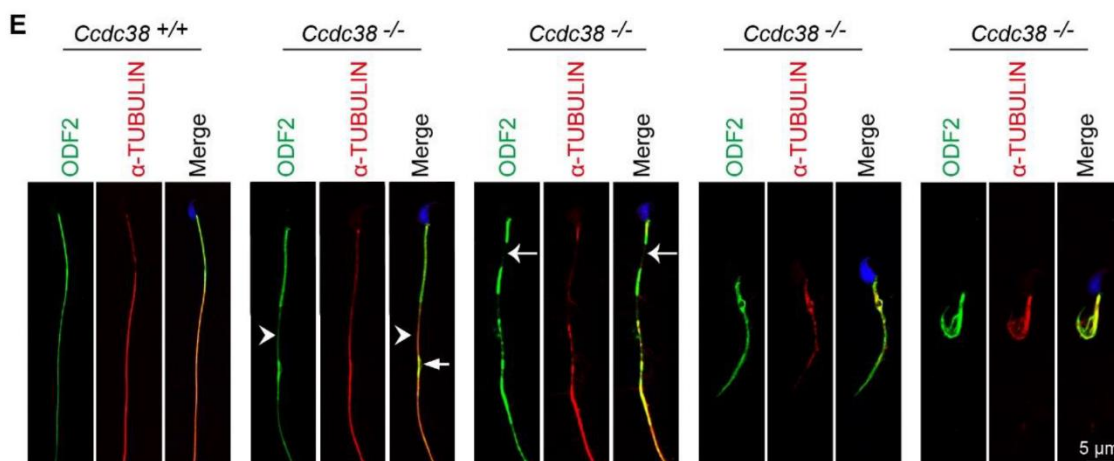


Fig 8. ODF transport is impaired in *Ccdc38* knockout spermatids.

(E) Immunofluorescence of ODF2 (green) and α -TUBULIN (red) in spermatids from *Ccdc38*^{+/+} and *Ccdc38*^{-/-} mice. Nuclei were stained with DAPI (blue). Short white arrows indicate disordered axoneme, long white arrows indicate a discontinuous, punctiform short axoneme, while white arrowheads indicate a tenuous axoneme. Data are presented as the mean \pm SD. Scale bars: 5 μ m.

13. There are many language errors in the text that need careful correcting the spelling, grammar, word use throughout this manuscript.

Response: Thank you for pointing out this problem. We have thoroughly revised the manuscript with the help of a native English speaker.

Second decision letter

MS ID#: DEVELOP/2022/200516

MS TITLE: Ccdc38 is required for sperm flagellum biogenesis and male fertility in mice

AUTHORS: Ruidan Zhang, Wei Li, Li Yuan, Fei Gao, Bingbing Wu, Chao Liu, Xiuge Wang, Liying Wang, Sai Xiao, Yinghong Chen, Huafang Wei, Zhe Zhang, and Hui Jiang

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 have addressed all my concerns. I do have no further points.

Comments for the author

The authors have addressed all my concerns. I do have no further points.

Reviewer 2

Advance summary and potential significance to field

The authors have now successfully addressed my concerns. I approve this manuscript for publication.

Comments for the author

The authors have now successfully addressed my concerns. I approve this manuscript for publication.