C2CD6 regulates targeting and organization of the CatSper calcium channel complex in sperm flagella

Fang Yang, Maria Gracia Gervasi, N. Adrian Leu, Gerardo Orta, Darya A. Tourzani, Jose Luis De la Vega-Beltrán, Gordon Ruthel, Alberto Darszon, Pablo E. Visconti and P. Jeremy Wang
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MS TITLE: C2CD6 is required for assembly of the CatSper calcium channel complex and fertilization

AUTHORS: Fang Yang, Maria Gracia Gervasi, N. Adrian Leu, Darya A Tourzani, Gordon Ruthel, Pablo E Visconti, and Jeremy Wang

I have now received the referees' reports on the above manuscript, and have reached a decision. As you will see, the referees express considerable interest in your work, but 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.

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

Yang et al. describe the role of C2CD6 in mouse sperm cells, a previously uncharacterized protein. The authors suggest that C2CD6 is part of the CatSper complex and/or necessary for the proper assembly of the CatSper complex into the flagellar membrane. Using C2CD6-KO mice they show

that lack of C2CD6 interferes with the formation of CatSper-nanodomains, inhibits capacitation-induced hyperactivation, and results in in male sterility, both in vivo and in vitro. Treatments with ionophore or starvation can rescue this phenotype to some extent. The study parts fit well together and address the putative function(s) of C2CD6 one step at a time in a logical manner. The findings described here for C2CD6 could be of potential high significance to the field, however, I do not find that the data presented here fully support all of the conclusions made, especially those concerning C2CD6 roles as a CatSper-subunit and/or as a facilitator of the transfer of CatSper-complexes to the flagellar membrane, see comments below.

Comments for the author

Essential revisions:
I think the authors should focus more on the important, novel, and interesting findings for C2CD6, where they have full support from their data, i.e. male sterility phenotype and inability to undergo capacitation-induced hyperactivation (Figure 3). The data presented in Figure 4+5 are of course interesting, but in my opinion these data do not fully support the claims made about C2CD6 putative roles as a novel subunit in CatSper and/or that C2CD6 should be essential for the incorporation of CatSper into the flagellar membrane. This is a major limitation of this manuscript. Additional experiments would be needed to draw such conclusions or alternatively to identify the other roles of C2CD6 responsible for the observed phenotype, see specific comments below:

- The authors claim that C2CD6 is part of the CatSper-complex in mouse sperm cells. However, in a recently published Cryo-EM paper on in Nature (doi: 10.1038/s41586-021-03742-6), which solved the structure of the so-called “CatSpermasome” in mouse sperm cells, this protein was not identified. Given this background information, the authors should either change their conclusions or argue why C2CD6 was not found in the Cryo-EM CatSpermasome?

- The authors claim that C2CD6 may be a functional subunit of CatSper. However, they only examine CatSper-related effects indirectly, e.g. via hyperactivated motility. The authors should have included electrophysiological measurements of CatSper-currents in the C2CD6-KO mouse sperm cells, to be able to state this. In figure 4 A+B, it seems clear that some CatSper1-staining is still seen in the C2CD6-KO sperm which I assume could still be part of functional CatSper-complexes?

- Also, the authors claim that C2CD6 is essential for the incorporation of the CatSper-complexes into the flagellar membrane, which seems more probable from the data. However, if C2CD6 was fully required for CatSper-complex incorporation into the flagellar membrane, how do the authors explain the CatSper1 staining still seen in Fig. 4A+B? It would perhaps be more correct to state that C2CD6 seems to be facilitating this incorporation, rather than being absolutely required.

- Furthermore, even though C2CD6 staining is much reduced in Fig. 4G, I do not think that this is evidence that C2CD6 colocalizes with CatSper1 and/or is an essential part of the CatSper complex. It may be that both proteins facilitate the transfer of the other to the flagellar membrane, without having any functional relationship there. Furthermore, the same reduction in expression was recently reported for Cdc42 (DOI: 10.1096/fj.202002773RR) and this protein was also not found as part of the Cryo-EM CatSpermasome in the Nature-paper.

- Although I can see why the authors are fond of the idea that C2CD6 is primarily regulating CatSper, as CatSper indeed is required for hyperactivated motility, I do not see why C2CD6 alone could not have other equally important roles in sperm and also be required for hyperactivated motility for multiple other reasons which do not necessarily have to do with CatSper, e.g. due to effects on capacitation? As you state, it was shown in Navarrete 2016, that ionophore treatment could reverse the sterile phenotypes of multiple KO mice, and not just of CatSper-KO mice. Furthermore, Navarrete 2019 could not rescue CatSper-KO with SER, but here you can indeed do that with C2CD6-KO sperm? If both proteins were really functional parts of the same CatSper ion channel complex, I do not see why such a difference should be observed?

- Super resolution microscopy: To be able to determine if C2CD6 co-localizes with CatSper1 in the quadrilateral nanodomains, the authors would have to use different antibody stains to do co-

staining of C2CD6 and CatSper1 in the same sperm cell samples. The images in Fig. 4B alone are not solid evidence for this.

- The presentation of co-immunoprecipitation data in Figure 5 is hard to follow. More explanation in the legend, method section and main text would have been nice, especially for researchers like me who have not done these types of experiments before. When looking up the technique its stated that co-immunoprecipitation "does not guarantee that the precipitated protein complexes are two proteins that interact directly". This should also be mentioned. I also think it should be noted that these co-immunoprecipitation experiments were performed in cells where CatSper-complexes are yet to be functionally expressed, as the authors state themselves, which I assume could mean that the CatSper-subunits behave differently here. Furthermore, I assume that both C2CD6 and the CatSper-subunits were overexpressed in these cells, increasing the likelihood that these exact proteins could find each other and interact, compared to in cells with regular expression-levels? Taken together, I think these Co-IP results should thus be assessed with caution.

Additional minor comments.
- Line 112-119: The authors should state how they validated these C2CD6-antibodies. They actually perform such validation in their C2CD6-KO sperm cells, in figure 1D.
- Line 184-185: It is unclear why they used 20uM ionophore for treatment 2, but only 5uM for treatment 4. Why did they use different concentrations? And how can they compare these conditions, as they do in Table 2? This information should also be found in the main text and table 2.
- Line 396-398: I agree much more with this statement, than the other conclusions. I think this should be the general conclusion, and the other "putative" functions related to CatSper-function should be downplayed.
- Line 453: I do not think this statement "C2CD6 is the newest CatSper subunit" is supported by the data.
- Figure 2 legend: (I) should be (E)
- Figure 4 legend: The real (E) is lacking, and (E)+(F) should be renamed to (F)+(G). Last sentence should be "immunofluorescence analysis of" rather than "localization of".
- Figure 2+3: It is unclear from the manuscript which statistical methods have been used and why this method was chosen?

Reviewer 2

Advance summary and potential significance to field

It is an interesting work identifying C2CD6 as a novel and essential subunit of the CatSper complex, in addition to the ten known subunits. The CatSper cation channel is essential for sperm capacitation and male fertility. Authors postulate that without C2CD6, the CatSper complex is not fully assembled in the flagellum and results in male sterility. C2CD6-deficient sperm are defective in hyperactivation and fail to fertilize oocytes both in vitro and in vivo. However there are several points that the authors should consider to improve the paper.
CatSper channel has been proposed as a target for male contraception with minimal side effects, The identification of C2CD6 might facilitate successful development of a heterologous CatSper system, which is not only critical for drug development but also provide a good system to dissect the mechanistic role of each subunit in CatSper.

Comments for the author

Major points:
- Authors postulated that without C2CD6, CatSper complex is not fully assembled. Indeed, the title highlights that postulation. They showed decrease in CatSper 1 expression in C2CD6 KO spermatozoa. There is no evidence in the manuscript showing wrong CatSper assembly. What about the other subunits? Please provide evidence of the deficiency in CatSper assembly or change the title.

- Authors should study the expression of C2CD6 in other organs of the male reproductive tract, not only the testis to affirm that C2CD6 is exclusive expressed in testis. And how is the epididymis and vas deferent of the KO mice?
Please show the entire blot of Figure 1C.
- It is known that human CatSper activity is different between epididymal sperm and ejaculated sperm. Does C2CD6 localization change between testicular sperm, caput sperm, cauda sperm and ejaculated sperm?
- Can C2CD6 KO sperm fuse zona-free eggs and zona-intact eggs (without cumulus)?
- How is the CatSper activity in the C2CD6 KO sperm? And the calcium levels before and after capacitation? Authors should measure tyrosine and PKA substrate phosphorylation levels after capacitation in the C2CD6 KO sperm.
- Authors should show co-localization of C2CD6 and CatSper by super-resolution or by confocal microscopy. Authors should perform proximity ligation assay or any other technique to show direct interaction between any subunit of CatSper and C2CD6. Co-ip shows interaction but it does not define if it is direct or indirect. Authors should provide the quantification of all Figure 5. That figure showed interaction between subunit of CatSper and C2CD6, but not direct interaction. Authors should study if there is direct interaction between these proteins. In HEK293T, is CatSper functional, is it fold correctly?
- Did the treatments with SER and ionophore also recover hyperactivated motility level in C2CD6 KO sperm?

Minor points:
- Please provide videos of sperm after capacitation showing the decrease in hyperactivated motility in KO sperm.
- Please for Figure 4 A, B, C, D, E, F and G, provide more examples. Authors should add quantification of the blots.
- Is C2CD6 present in human sperm? Does it interact with human CatSper? Is it known any mutation of C2CD6 that affects human fertility?
- It has been described different inhibitors of CatSper, some are proteins such as CRISP1. Does C2CD6 bind to this protein or other ones?
- Is C2CD6 regulated by alkaline pH? Is the interaction between C2CD6 and CatSper changes depending on the extracellular pH? Does the efflux of K+ influence in the interaction between C2CD6 and CatSper?

First revision

Author response to reviewers' comments

Thank you for the opportunity to submit a revised manuscript. We thank both reviewers for their considerable interest in this study and their thoughtful comments. We have revised the manuscript substantially. We have addressed both reviewers' points with a substantial amount of new experimental data and text revisions. We added one new figure and three new supplementary figures. We feel that this new version is considerably stronger. New or revised texts are shown in magenta. When the entire sections in Results or Materials & Methods are new, only subheadings are shown in magenta for the convenience of reading.

Please note that three new authors have been added: Gerardo Orta, Jose Luis de la Vega-Beltrán, and Alberto Darszon, because they have contributed new experimental data (Figure 5 and Figure S3) for the revision. All authors have agreed with these additions.

Here are the main new data:
Figure 5: Electrophysiology. Our new data showed that the CatSper channel is functional but at a significantly reduced capacity in *C2cd6*$^{-/-}$ cauda sperm. We have been trying to ship mice to Dr. Alberto Darszon's lab for electrophysiology at National University of Mexico for about one year. It was repeatedly delayed due to various pandemic-related restrictions. Finally, the mice were shipped and Dr. Alberto Darszon and colleagues were able to perform the patch clamp and albumin treatment experiments.

Figure S1. Expression and localization of C2CD6 in reproductive tissues and sperm.
Figure S2. Analysis of PKA phosphorylation and tyrosine phosphorylation in sperm before and after capacitation.
Figure S3. BSA induces [Ca2+]i (calcium influx) increase in mouse sperm.

Below is our point-by-point response to the reviewers' comments.

Reviewer 1 Advance Summary and Potential Significance to Field: Yang et al. describe the role of C2CD6 in mouse sperm cells, a previously uncharacterized protein. The authors suggest that C2CD6 is part of the CatSper complex and/or necessary for the proper assembly of the CatSper complex into the flagellar membrane. Using C2CD6-KO mice they show that lack of C2CD6 interferes with the formation of CatSper-nanodomains, inhibits capacitation- induced hyperactivation, and results in male sterility, both in vivo and in vitro. Treatments with ionophore or starvation can rescue this phenotype to some extent. The study parts fit well together and address the putative function(s) of C2CD6 one step at a time in a logical manner. The findings described here for C2CD6 could be of potential high significance to the field, however, I do not find that the data presented here fully support all of the conclusions made, especially those concerning C2CD6 roles as a CatSper-subunit and/or as a facilitator of the transfer of CatSper- complexes to the flagellar membrane, see comments below.

Reviewer 1 Comments for the Author:
Essential revisions:

I think the authors should focus more on the important, novel, and interesting findings for C2CD6, where they have full support from their data, i.e. male sterility phenotype and inability to undergo capacitation-induced hyperactivation (Figure 3). The data presented in Figure 4+5 are of course interesting, but in my opinion these data do not fully support the claims made about C2CD6 putative roles as a novel subunit in CatSper and/or that C2CD6 should be essential for the incorporation of CatSper into the flagellar membrane. This is a major limitation of this manuscript. Additional experiments would be needed to draw such conclusions or alternatively to identify the other roles of C2CD6 responsible for the observed phenotype, see specific comments below:
Response: We agree. We have revised the manuscript accordingly. In addition, our new patch clamp data provided a substantially amount of new information.

•The authors claim that C2CD6 is part of the CatSper-complex in mouse sperm cells. However, in a recently published Cryo-EM paper on in Nature (doi: 10.1038/s41586-021-03742-6), which solved the structure of the so- called "CatSpermasome" in mouse sperm cells, this protein was not identified. Given this background information, the authors should either change their conclusions or argue why C2CD6 was not found in the Cryo-EM CatSpermasome?

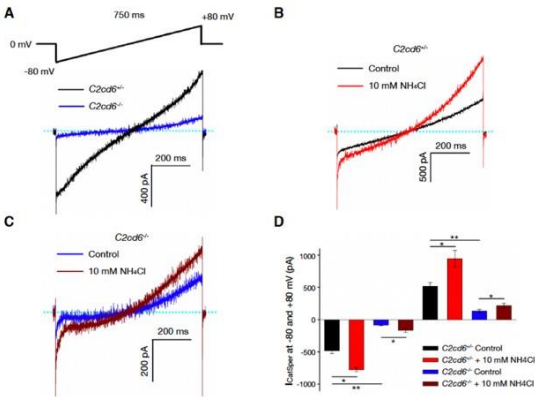
Response: In this study (Extended data Fig. 2a, Lin et al., Nature, 2021), C2CD6 was actually identified in the CatSpermasome by mass spectrometric analysis. C2CD6 has 14 peptides identified and has more peptides identified than CatSper1, 2, 3, z, and EFCAB9 in the mass spec analysis. However, C2CD6 was not delineated in the Cryo-EM structure of the CatSpermasome. There are several extra densities in the Cryo-EM structure, which corresponded to the uncharacterized components (Fig. 1a, b; Lin et al., Nature, 2021). It is highly likely that C2CD6 and several other new components are present in the unsolved extra densities in the CatSpermasome. We have now discussed this point in the revision (2nd paragraph in Discussion).

•The authors claim that C2CD6 may be a functional subunit of CatSper. However, they only examine CatSper-related effects indirectly, e.g. via hyperactivated motility. The authors should have included electrophysiological measurements of CatSper-currents in the C2CD6-KO mouse sperm cells, to be able to state this. In figure 4 A+B, it seems clear that some CatSper1-staining is still seen in the C2CD6-KO sperm, which I assume could still be part of functional CatSper-complexes?
Response: Thank the reviewer for this suggestion! In collaboration with Alberto Darszon's lab, we have performed electrophysiological measurements of CatSper currents in *C2cd6* KO sperm and *C2cd6*$^{+/-}$ (control) sperm. We detected CatSper current in *C2cd6* KO sperm, which is significantly

lower than that in *C2cd6*$^{+/-}$ sperm. Furthermore, the current is stimulated with NH4Cl, a well-defined property of CatSper channels. Contrary to the CatSper1 KO, these new data show that, although with reduced activity, the CatSper channel is functional in *C2cd6* KO sperm. This is consistent with the presence of CatSper in the C2cd6 KO sperm flagella revealed by immunofluorescence.

Altogether, these results suggest that C2CD6 is important for targeting and organization of the assembled CatSper channel complex in the sperm flagella rather than the assembly of the channel complex. We have added these data in a new figure: Figure 5, added a paragraph in Results, and revised the title. Moreover, in supplemental Fig. S3, we showed that the CatSper-dependent BSA-induced increase in intracellular calcium is significantly reduced in the *C2cd6* KO sperm.

Figure 5.



•Also, the authors claim that C2CD6 is essential for the incorporation of the CatSper-complexes into the flagellar membrane, which seems more probable from the data. However, if C2CD6 was fully required for CatSper-complex incorporation into the flagellar membrane, how do the authors explain the CatSper1 staining still seen in Fig. 4A+B? It would perhaps be more correct to state that C2CD6 seems to be facilitating this incorporation, rather than being absolutely required.
Response: We agree with this interpretation. The new patch clamp data (Figure 5) further supported this interpretation. We have revised the manuscript as suggested. Our data strongly suggest that C2CD6 plays a major role in facilitating this incorporation. We also discussed the possibility that other proteins may play a minor role.

•Furthermore, even though C2CD6 staining is much reduced in Fig. 4G, I do not think that this is evidence that C2CD6 colocalizes with CatSper1 and/or is an essential part of the CatSper complex. It may be that both proteins facilitate the transfer of the other to the flagellar membrane, without having any functional relationship there.
Furthermore, the same reduction in expression was recently reported for Cdc42 (DOI: 10.1096/fj.202002773RR) and this protein was also not found as part of the Cryo-EM CatSpermasome in the Nature-paper.
Response: We appreciate this suggestion. We agree and have revised accordingly. C2CD6 is present in the CatSpermasome. We find that C2CD6 is associated with CatSper core subunits. We agree that both proteins facilitate the transfer of each other to the flagella. The CDC42 study is very informative. We have discussed CDC42 in the Discussion.

•Although I can see why the authors are fond of the idea that C2CD6 is primarily regulating CatSper, as CatSper indeed is required for hyperactivated motility, I do not see why C2CD6 alone could not have other equally important roles in sperm and also be required for hyperactivated motility for multiple other reasons, which do not necessarily have to do with CatSper, e.g. due to effects on capacitation? As you state, it was shown in Navarrete 2016, that ionophore treatment could reverse the sterile phenotypes of multiple KO mice, and not just of CatSper-KO mice. Furthermore, Navarrete 2019 could not rescue CatSper-KO with SER, but here you can indeed do that with C2CD6- KO sperm? If both proteins were really functional parts of the same CatSper ion channel complex, I do not see why such a difference should be observed?
Response: We agree with this alternative interpretation. Another explanation to consider is that the CatSper1 KO sperm lacks completely the CatSper channel complex. In the *C2cd6* KO sperm,

although the activity is reduced, the CatSper channel is still functional. We have revised the discussion accordingly by adding these possibilities in Discussion: “It could also be due to the presence of residual functional CatSper channels in C2CD6-deficient sperm. Another possible explanation for the difference in the SER treatment response is that C2CD6 could regulate capacitation independent of CatSper.”

•Super resolution microscopy: To be able to determine if C2CD6 co-localizes with CatSper1 in the quadrilateral nanodomains, the authors would have to use different antibody stains to do co-staining of C2CD6 and CatSper1 in the same sperm cell samples. The images in Fig. 4B alone are not solid evidence for this.
Response: Our CatSper1 antibody is rabbit. We tried to raise anti-C2CD6 antibodies in guinea pig but the guinea pig antibody did not work. Therefore, we could not perform super resolution microscopy of the double immunostaining.
An independent study by Hwang et al from Jean-Ju Chung’s lab also reported the quadrilateral nanodomains of C2CD6 (bioRxiv 2021.08.16.456347; doi: https://doi.org/10.1101/2021.08.16.456347). This preprint was deposited at bioRxiv 40 days after the release of our preprint (bioRxiv 2021.07.06.451342; doi: https://doi.org/10.1101/2021.07.06.451342). Chung lab did not perform double staining either, possibly due to lack of compatible antibodies. Their result is consistent with ours. Since Chung’s preprint did not cite our preprint (which was posted 40 days before theirs), we are not sure about the journal’s policy on whether we should cite a preprint. If Dr. Arur and Reviewers ask us to cite a preprint, we will be happy to do so.

•The presentation of co-immunoprecipitation data in Figure 5 is hard to follow. More explanation in the legend, method section and main text would have been nice, especially for researchers like me who have not done these types of experiments before. When looking up the technique its stated that co-immunoprecipitation “does not guarantee that the precipitated protein complexes are two proteins that interact directly”. This should also be mentioned. I also think it should be noted that these co-immunoprecipitation experiments were performed in cells where CatSper-complexes are yet to be functionally expressed, as the authors state themselves, which I assume could mean that the CatSper-subunits behave differently here. Furthermore, I assume that both C2CD6 and the CatSper-subunits were overexpressed in these cells, increasing the likelihood that these exact proteins could find each other and interact, compared to in cells with regular expression-levels? Taken together, I think these Co-IP results should thus be assessed with caution.
Response: Co-transfection and co-expression have been routinely used for studying protein-protein interactions in many studies including the EFCAB9 study (Hwang et al., 2019). This type of experiments test whether two proteins are associated with each other. Although the association of two proteins could be bridged by an endogenous protein, this has rarely been the case. 293T cells are routinely used, because of high transfection efficiency. Because C2CD6 and CatSper subunits are not expressed endogenously in 293T cells, co-expression in 293T cells allow to test their association. Indeed, these caveats need to be taken into consideration for data interpretation and these co-IP results need to be assessed with caution. Importantly, our co-IP results are consistent with the co-IP results from testes performed by Jean-Ju Chung’s lab in their preprint on C2CD6 (bioRxiv 2021.08.16.456347; doi: https://doi.org/10.1101/2021.08.16.456347). We have revised the Results section accordingly. We have described in Figure legend in detail.

Additional minor comments.
•Line 112-119: The authors should state how they validated these C2CD6-antibodies. They actually perform such validation in their C2CD6-KO sperm cells, in figure 1D.

Response: We have described the specificity of this antibody (3rd paragraph of the Results section), since the signal is absent in KO sperm.

•Line 184-185: It is unclear why they used 20 uM ionophore for treatment 2, but only 5 uM for treatment 4. Why did they use different concentrations? And how can they compare these conditions, as they do in Table 2? This information should also be found in the main text and table 2.
Response: We previously reported that when used alone, 20 μM of ionophore induced the desired effect on sperm motility and fertilization results. However, when used in combination with the SER

treatment, 20 μM ionophore was detrimental to the sperm sample, sperm did not recover well, and therefore, the concentration of ionophore was decreased to 5 μM for treatment 4. We have added this information to the text and Table 2.

•Line 396-398: I agree much more with this statement, than the other conclusions. I think this should be the general conclusion, and the other "putative" functions related to CatSper-function should be downplayed.
Response: We have emphasized the role in CatSper targeting to flagellar membrane as the general conclusion and downplayed the CatSper channel-related functions.

•Line 453: I do not think this statement "C2CD6 is the newest CatSper subunit" is supported by the data.
Response: Deleted.

•Figure 2 legend: (I) should be (E)
Response: Corrected! Sorry for the oversight!

•Figure 4 legend: The real (E) is lacking, and (E)+(F) should be renamed to (F)+(G). Last sentence should be "immunofluorescence analysis of" rather than "localization of".
Response: Corrected! We appreciate your careful effort and apologize for this oversight!

•Figure 2+3: It is unclear from the manuscript which statistical methods have been used and why this method was chosen?
Response: We have added statistical method (Student's *t*-test) to the figure legends.

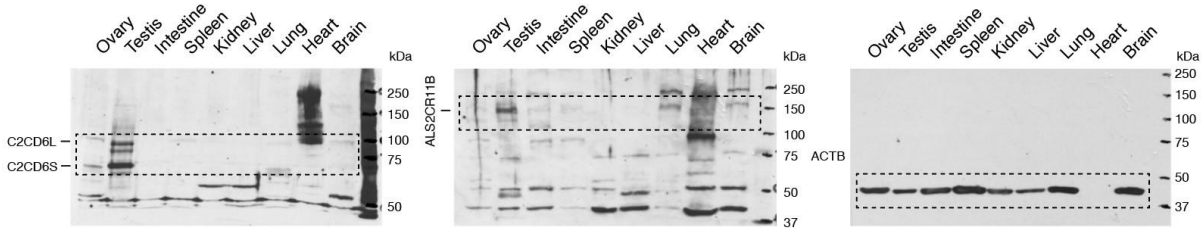
Reviewer 2 Advance Summary and Potential Significance to Field:
It is an interesting work identifying C2CD6 as a novel and essential subunit of the CatSper complex, in addition to the ten known subunits. The CatSper cation channel is essential for sperm capacitation and male fertility. Authors postulate that without C2CD6, the CatSper complex is not fully assembled in the flagellum and results in male sterility. C2CD6-deficient sperm are defective in hyperactivation and fail to fertilize oocytes both in vitro and in vivo. However, there are several points that the authors should consider to improve the paper. CatSper channel has been proposed as a target for male contraception with minimal side effects, The identification of C2CD6 might facilitate successful development of a heterologous CatSper system, which is not only critical for drug development but also provide a good system to dissect the mechanistic role of each subunit in CatSper.

Reviewer 2 Comments for the Author:
Major points:

-Authors postulated that without C2CD6, CatSper complex is not fully assembled. Indeed, the title highlights that postulation. They showed decrease in CatSper 1 expression in C2CD6 KO spermatozoa. There is no evidence in the manuscript showing wrong CatSper assembly. What about the other subunits? Please provide evidence of the deficiency in CatSper assembly or change the title.
Response: We appreciate this suggestion. Our patch clamp data in C2CD6 knockout sperm showed that the CatSper channel function is intact. We have revised the title accordingly.

-Authors should study the expression of C2CD6 in other organs of the male reproductive tract, not only the testis to affirm that C2CD6 is exclusive expressed in testis. And how is the epididymis and vas deferent of the KO mice?
Please show the entire blot of Figure 1C.
Response: We have now studied the expression of C2CD6 in epididymis and vas deferens from day-26 wild-type and *C2cd6*$^{-/-}$ males. Day 26 was chosen, because the epididymis at this age lacks sperm. C2CD6 is not expressed in epididymis or vas deferens. These new data are shown in a new supplementary figure – Figure S1A.

The three entire WB blots for Figure 1C are shown below for review purpose only. If the reviewer and Dr. Arur think that this should be included as a supplementary figure, we will be happy to do so.



Entire blots for Figure 1C. Dashed boxed regions are cropped into Figure 1C.

-It is known that human CatSper activity is different between epididymal sperm and ejaculated sperm. Does C2CD6 localization change between testicular sperm, caput sperm, cauda sperm and ejaculated sperm?
Response: We have now examined the localization of C2CD6 in testicular sperm, caput sperm and cauda sperm. We didn't use ejaculated sperm; in the mouse, this collection is difficult because ejaculated sperm are immediately coagulated after forming a plug. We have also examined the localization of CatSper1 for comparisons. C2CD6 and CatSper1 are not detected in the growing flagella in step 8 testicular spermatids. They are present in the principal piece of testicular sperm at step 9/10 and beyond, caput sperm, and cauda sperm. These new data are shown in Figure S1A and S1B.

-Can C2CD6 KO sperm fuse zona-free eggs and zona-intact eggs (without cumulus)?
Response: We have not done this experiment, because our results using IVF show that upon a temporary increase in intracellular calcium either by the use of ionophore, by starvation, or by a combination of both treatments, *C2cd6* KO sperm are able to fertilize, indicating their ability to fuse.

-How is the CatSper activity in the C2CD6 KO sperm? And the calcium levels before and after capacitation? Authors should measure tyrosine and PKA substrate phosphorylation levels after capacitation in the C2CD6 KO sperm.
Response: Thank the reviewer for this suggestion! In collaboration with Alberto Darszon's lab, we have performed electrophysiological measurements of CatSper currents in *C2cd6* KO sperm and *C2cd6*$^{+/-}$ (control) sperm. We detected CatSper current in *C2cd6* KO sperm, which is significantly lower than that in *C2cd6*$^{+/-}$ sperm. Furthermore, the current is stimulated with NH_4Cl, a well-defined property of CatSper channels. Contrary to the CatSper1 KO, these new data show that, although with reduced activity, the CatSper channel is functional in *C2cd6* KO sperm. This is consistent with the presence of CatSper in the C2cd6 KO sperm flagella revealed by immunofluorescence.
Altogether, these results suggest that C2CD6 is important for targeting and organization of the assembled CatSper channel complex in the sperm flagella rather than the assembly of the channel complex. We have added these data in a new figure: Figure 5, added a paragraph in Results, and revised the title. Moreover, in supplemental Fig. S3, we showed that the CatSper-dependent BSA-induced increase in intracellular calcium is significantly reduced in the *C2cd6* KO sperm.

We have also measured tyrosine and PKA substrate phosphorylation levels after capacitation in the C2CD6 KO sperm. These new data are presented in a new supplementary figure – Figure S2. We have described these new results as follows: Calcium influx regulates PKA phosphorylation and protein tyrosine phosphorylation in sperm upon capacitation. The phosphorylation of PKA substrates was diminished in sperm from *C2cd6*$^{-/-}$ males in comparison with *C2cd6*$^{+/-}$ males (Fig. S2A). However, the increase in tyrosine phosphorylation that occurs during sperm capacitation was similar between *C2cd6*$^{-/-}$ sperm and *C2cd6*$^{+/-}$ sperm (Fig. S2B). Considering that PKA activation (measured by Western blotting analyses of phosho-PKA substrates and tyrosine phosphorylation) have a biphasic dependence on calcium (Navarrete et al. J. of Cell Physiol. 2015), these results are consistent with the reduced functionality of CatSper channels in *C2cd6*-deficient sperm.

-Authors should show co-localization of C2CD6 and CatSper by super-resolution or by confocal microscopy. Authors should perform proximity ligation assay or any other technique to show direct interaction between any subunit of CatSper and C2CD6. Co-ip shows interaction but it does not define if it is direct or indirect. Authors should provide the quantification of all Figure 5. That figure showed interaction between subunit of CatSper and C2CD6, but not direct interaction. Authors should study if there is direct interaction between these proteins. In HEK293T, is CatSper functional, is it fold correctly?
Response: Our CatSper1 antibody is rabbit. We tried to raise anti-C2CD6 antibodies in guinea pig but the guinea pig antibody did not work. Therefore, we could not perform super resolution microscopy of the double immunostaining.
An independent study by Hwang et al from Jean-Ju Chung's lab also reported the quadrilateral nanodomains of C2CD6 (bioRxiv 2021.08.16.456347; doi: https://doi.org/10.1101/2021.08.16.456347). This preprint was deposited at bioRxiv 40 days after the release of our preprint (bioRxiv 2021.07.06.451342; doi: https://doi.org/10.1101/2021.07.06.451342). Chung lab did not perform double staining either, possibly due to lack of compatible antibodies. Their result is consistent with ours.

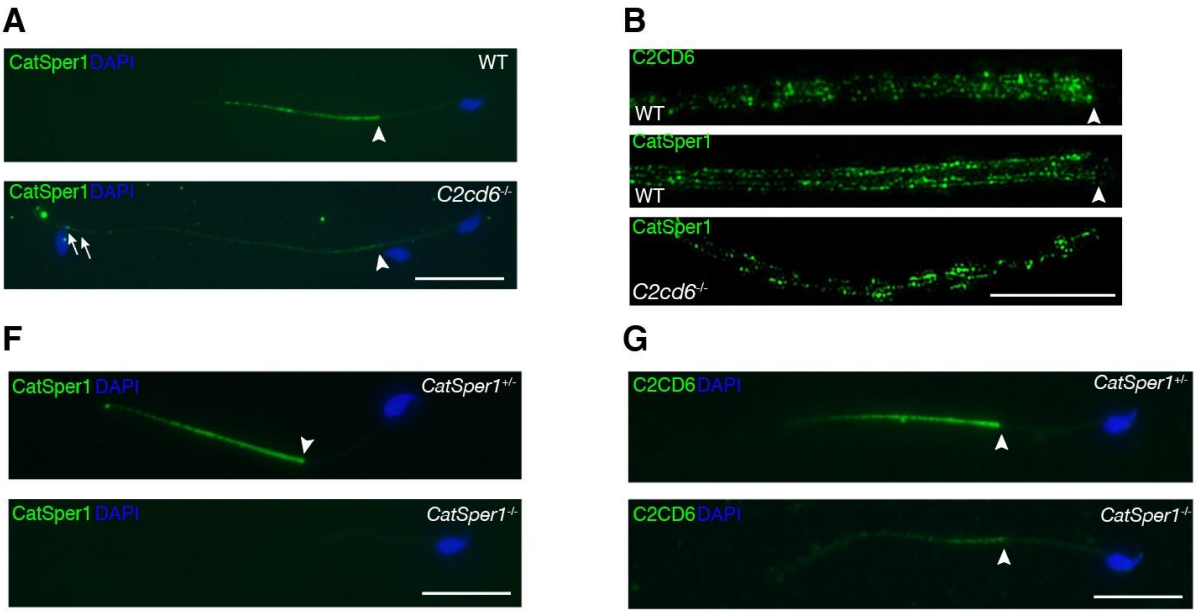
Figure 5 is now Figure 6. Co-IP results are qualitative but not quantitative. Quantification will not be informative. In some cases, C2CD6 was not detected in the input or present in very low level. To be more informative, we have described in more detail the experimental design. Input is 1% of the protein extracts used for IP. Demonstration of direct interaction requires expression and purification of recombinant proteins, which will take years. Therefore, we think that proximity ligation assay and demonstration of direct interactions are important but beyond the scope of the current study.

-Did the treatments with SER and ionophore also recover hyperactivated motility level in C2CD6 KO sperm? **Response**: We tested the effect of these treatments on fertilization but not on the hyperactivated motility level in KO sperm. Because hyperactivation is essential for fertilization, our results suggest that hyperactivation is rescued with the SER and ionophore treatments. These treatments were done in UMass Amherst, but the mice are kept at UPenn (Philadelphia). The new transfer of mice takes a long time and we were not able to perform these experiments within the revision time frame.

Minor points:

-Please provide videos of sperm after capacitation showing the decrease in hyperactivated motility in KO sperm. **Response:** The videos recorded by the CASA system are very short and are difficult to appreciate changes in sperm hyperactivation by naked eye. If the reviewer and the editor require them, we can provide these videos but we don't think they are informative in this format. However, CASA did provide quantification of hyperactivity (Fig. 3).

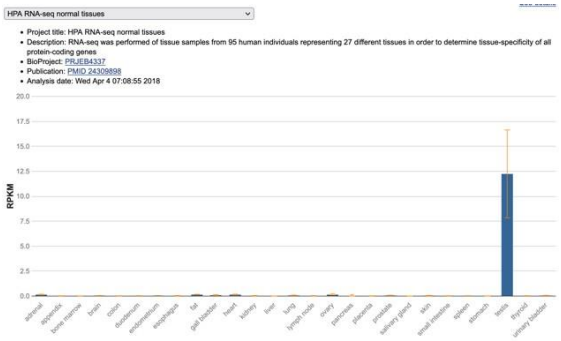
-Please for Figure 4 A, B, C, D, E, F and G, provide more examples. Authors should add quantification of the blots. **Response**: We added quantification of band intensities (C, D, E) to the Western blots. Representative IF images are shown in Fig. 4. We have many more images. Here we prepared an additional set of images for Figure 4A, B, F, and G (see the attached figure below). We don't feel that it is necessary to show these additional images as a supplementary figure. If the Reviewers and Dr. Arur ask us to include it as a supplementary figure, we will be happy to do so.



Additional images (Fig. 4) for reviewing purpose

(A) Immunofluorescence analysis of CatSper1 in wild type and *C2cd6*-deficient sperm. Arrowhead indicates the annulus. Scale bar, 25 μm.
(B) Super-resolution localization of C2CD6 and CatSper1 in wild type and *C2cd6*$^{-/-}$ sperm. The distal region of the flagellar principal piece of the *C2cd6*$^{-/-}$ sperm is shown. Scale bar, 5 μm.
(F) Immunofluorescence analysis of CatSper1 in *CatSper1*$^{+/-}$ and *CatSper1*$^{-/-}$ sperm from adult males. Scale bar, 25 μm.
(G) Immunofluorescence analysis of C2CD6 in *CatSper1*$^{+/-}$ and *CatSper1*$^{-/-}$ sperm from adult males. Scale bar, 25 μm.

-Is C2CD6 present in human sperm? Does it interact with human CatSper? Is it known any mutation of C2CD6 that affects human fertility?
Response: C2CD6 is conserved in humans. And according to NCBI database, this protein is also testis-specific in humans (https://www.ncbi.nlm.nih.gov/gene/151254) (See the human RNA-seq data graph on the right). In the preprint from Jean-Ju Chung's lab (bioRxiv 2021.08.16.456347; doi: https://doi.org/10.1101/2021.08.16.456347), they showed that C2CD6 localizes to the principal piece of human ejaculated sperm (Figure 1E, Hwang et al., bioRxiv). We agree that human studies will be interesting but beyond the scope of the current study.



-It has been described different inhibitors of CatSper, some are proteins, such as CRISP1. Does C2CD6 bind to this protein or other ones?
Response: We have not been able to test these. C2CD6 but not CRISP1 was identified in the CatSpermasome (Lin et al., Nature 2021). These would be interesting future studies.

-Is C2CD6 regulated by alkaline pH? Is the interaction between C2CD6 and CatSper changes depending on the extracellular pH? Does the efflux of K+ influence in the interaction between C2CD6 and CatSper?
Response: We agree that these would be very interesting biochemical studies in the future. This would require production of recombinant proteins, which would constitute a separate study.

I hope that the revised manuscript is now suitable for publication. Sincerely yours,
Jeremy Wang

Second decision letter

MS ID#: DEVELOP/2021/199988

MS TITLE: C2CD6 regulates targeting and organization of the CatSper calcium channel complex in sperm flagella

AUTHORS: Fang Yang, Maria Gracia Gervasi, N. Adrian Leu, Gerardo Orta, Darya A Tourzani, Jose Luis De la Vega-Beltran, Gordon Ruthel, Alberto Darszon, Pablo E Visconti, and Jeremy Wang

I have now received all the referees reports on the above manuscript, and have reached a decision. The overall evaluation is positive and we would like to publish a revised manuscript in Development. However, I agree with the reviewers that the preprint which is consistent with the findings of this paper should be cited. https://doi.org/10.1101/2021.08.16.456347 as well as the comment on more examples (if possible) of Figure 4 to be provided in the supplement. I expect to not send the paper back to the reviewers once I receive the revision with the appropriate citation and a response to reviewers. Please do highlight all the changes made in the manuscript.

Reviewer 1

Advance summary and potential significance to field

The authors addressed all of my comments in a satisfactory manner. I think the manuscript has much improved.

Comments for the author

My final comment is that I personally think the authors should mention the pre-print from Jean-Ju Chung's lab (https://doi.org/10.1101/2021.08.16.456347) in the discussion section, since these two studies on C2CD6 complement each other and present consistent results. I hope the journal's policy will make this possible.

Reviewer 2

Advance summary and potential significance to field

It is an interesting work identifying C2CD6 as a novel and essential subunit of the CatSper complex. The CatSper cation channel is essential for sperm capacitation and male fertility. Authors postulate C2CD6 is important for targeting and organization of the assembled CatSper channel complex in the sperm flagella. C2CD6-deficient sperm are defective in hyperactivation and fail to fertilize oocytes both in vitro and in vivo. CatSper channel has been proposed as a target for male contraception with minimal side effects. The identification of C2CD6 might facilitate successful development of a heterologous CatSper system which is not only critical for drug development but also provide a good system to dissect the mechanistic role of each subunit in CatSper.

Comments for the author

The authors have replied adequately the reviewer comments. For this reviewer is important to show in a supplementary figure more examples of Fig. 4.

Second revision

Author response to reviewers' comments

Thank you for considering our manuscript for publication!

I'd like to first address your summary points:

"I have now received all the referees reports on the above manuscript, and have reached a decision. The overall evaluation is positive and we would like to publish a revised manuscript in Development. However, I agree with the reviewers that the preprint which is consistent with the findings of this paper should be cited. https://doi.org/10.1101/2021.08.16.456347 as well as the comment on more examples (if possible) of Figure 4 to be provided in the supplement. I expect to not send the paper back to the reviewers once I receive the revision with the appropriate citation and a response to reviewers. Please do highlight all the changes made in the manuscript."

Response: We have revised the manuscript by addressing the above two points per your instruction. First, we have now cited the preprint in the discussion (Lines 286-287). Second, more examples of fluorescence images of Figure 4 have been included in a new supplementary figure – Fig. S3. Fig. S3 is described in Figure 4 legend (Lines 827-828). All the changes are highlighted in magenta.

Below is my point by point response to the reviews:

Reviewer 1 Comments for the Author:
My final comment is that I personally think the authors should mention the pre-print from Jean-Ju Chung's lab (https://doi.org/10.1101/2021.08.16.456347) in the discussion section, since these two studies on C2CD6 complement each other and present consistent results. I hope the journal's policy will make this possible.

Response: We have now cited the preprint in the discussion (Lines 286-287) by adding this description: "Our results are consistent with a preprint independent study by Hwang et al (Hwang et al., 2021)."

Reviewer 2 Comments for the Author:
The authors have replied adequately the reviewer comments. For this reviewer is important to show in a supplementary figure more examples of Fig. 4.

Response: More examples of fluorescence images of Figure 4 have been included in a new supplementary figure – Fig. S3. Fig. S3 is described in Figure 4 legend (Lines 827-828).

Third decision letter

MS ID#: DEVELOP/2021/199988

MS TITLE: C2CD6 regulates targeting and organization of the CatSper calcium channel complex in sperm flagella

AUTHORS: Fang Yang, Maria Gracia Gervasi, N. Adrian Leu, Gerardo Orta, Darya A Tourzani, Jose Luis De la Vega-Beltran, Gordon Ruthel, Alberto Darszon, Pablo E Visconti, and Jeremy Wang
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.