CCDC66 regulates primary cilium length and signaling via interactions with transition zone and axonemal proteins

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MS TITLE: CCDC66 regulates primary cilium length and signaling competence via multi-site interactions with transition zone and axonemal proteins

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript the authors reported CCDC66 as a new ciliary axoneme/tip protein and investigated its roles in cilium ciliogenesis and function regulation most significantly, the cilium length control. This paper to some extent provides insights into the mechanism by which ciliary MAPs cooperate to regulate the cilia structure and function

Comments for the author

I have some concerns or suggestions as followed:
1. When observing the localization of CCDC66 in vivo (for example, Figure 1A), the authors showed only GFP tagged CCDC66, but no GFP only control, how could exclude the possibility that the localization of GFP-CCDC66 in vivo is artefact caused by GFP itself?

2. In Figure 1F, the mScarlet-Arl13b was in a smear pattern with some granules in my opinion, mNG-CCDC66 and mScarlet-Arl13b didn't show significant co-localization until 536 min, but this co-localization was lost in 720 min (no or too weak mScarlet-Arl13b signal?), please explain.

3. The authors found the microtubule-binding fragment (570-948) was sufficient to localize CCDC66 to primary cilium, was it essential?

4.There are four axonemal proteins required for cilium length control including CSPP1, ARMC9, CEP104 and TOGARAM1, why only chose CSPP1 and CEP104?

5. As a supplement to Figure S4E, the authors should prepare a panel for verifying the siRNA knock down efficiency of CEP104 and CSPP1.

6. In CCDC66-depleted cells, Ciliary level of mNG-CEP104 increased (Figure 5E)
and expression of mNG-CEP104 compensated for the cilium length defects (Figure 5H), so how to interpret the relationship between increased ciliary localization and rescued cilium length defects? Is there a possibility that the increased ciliary localization of CEP104 promote cilium growth?

7. According to the IP data, the authors identified that CCDC66 interacted with CEP104 directly, in my opinion, to more straightforwardly support the proposal that 'CCDC66 might be involved in cilium length control via regulation CEP104-mediated axonemal polymerization'(page 15), if possible, it's better to generate a CCDC66 mutant that only disrupt the interaction with CEP104, then see what will happen when transfected to CCDC66-depleted cells.

Reviewer 2

Advance summary and potential significance to field

CCDC66 is important for ciliogenesis but it localises to many different ciliary associated structures. In this paper, the authors begin to dissect the function of CCDC66 at these different ciliary associated structures by using a set of mutants that have restricted localisations. This has provided important insights into the function of CCDC66 and how it contributes to ciliogenesis.

Comments for the author

Overall, this is a well written and easily followed manuscript. Generally, the data presented supports the conclusions drawn; however, the authors are prosecuting the argument that the CCDC66-CEP290 interaction is exclusively at the centriolar satellites but their data is not always solid for this.

Pg6 – the authors state that CCDC66 did not colocalise with CEP290 at the transition zone but based on the images presented there is clearly CCDC66 signal at the transition zone. It is not the region of brightest signal but definitely there and the authors need to acknowledge this. There is an inverse relationship between the CEP290 and CCDC66 signals but still overlap at the transition zone. Moreover, by eye the CCDC66 signal intensity at the centriolar satellites is similar to that at the transition zone.

Given the drop in CEP290 expression that occurs in the PCM1 KO cells, which the authors acknowledge, I would be a bit more circumspect based on the evidence in concluding the CCDC66-CEP290 interaction is satellite dependent. It is possible that the interaction first occurs there but it can occur in other places. As the CCDC66 signal overlaps with CEP290 at other points than the satellites. Plus the 'exclusive' localisation of CCDC66 to the centrosome was able to restore CEP290 levels at the transition zone, which suggests CCDC66 can interact with CEP290 at other locations than the centriolar satellites. Does the tethering of CDCC66 to the centrosome alter the localisation of other centriolar satellite proteins?
PCM1 looks unaffected but those images are from cells in which endogenous CCDC66 is expressed – on depletion is there a change in localisation?

Minor points

Pg5 - 'although no Joubert-causative CCDC66 mutations are hitherto not reported' – this doesn't make sense. I presume it means no mutations in CCDC66 have been reported?

The authors show that the amino acids 570-948 are sufficient to target mNG to the basal body/cilium but what happens when this region is deleted from CCDC66 – is it necessary for the protein localisation to these regions?

Pg7 – should this be mNG-CCDC66 not GFP-CCDC66 in the second section?

The time lapse examples clearly shows the assembly and disassembly of CCDC66 into the cilium but I was unsure how many movies these conclusions were based on. Can you add this detail into the figure legend.

In figure 2D, the mean cilium length for the knockdown cells looks to continue to gradual increase across the time course and not plateau at 200 min. The control rapidly increases in the first 200 mins before levelling out but the knockdown grows slowly throughout.

Pg11 – fwith

S3A – western blot of mNG expression is not very convincing, it is difficult to identify bands in comparison to the parental cells. What are the expected sizes of the fusion proteins?

Pg12 – given the western blot I think it is difficult to make strong conclusions about the expression levels of these constructs. Moreover, given that these cell lines are all generated using similar approaches it is not surprising the localisation of CCDC66 and its mutants are consistent but without the localisation of the endogenous protein this statement about artefacts is difficult to verify. Do the commercial antibodies work for westerns? This might help to give a handle of relative expression levels of the tagged protein and the endogenous protein.

5A/S4C – on the schematic the CCDC66 region 467-570 is highlighted and in the supplementary figure the mutant 1-570 weakly interacts with PCM1 and CSPP1 while the 1-408 mutant does not. What is significant about this region? It is not mentioned in the manuscript? Where does this construct localise?

Pg14 – In addition ot

In the experiments depleting CCDC66 while expressing mNG-CEP104 etc was there any change in the number ciliated cells or was just the length affected?

Fig. 5J – the authors say that CEP104 RNAi further reduced cilium length but it's not clear what they mean. Further from what? Are they suggesting some synergistic effect?

6H – there is a reduction in the ciliary tip localisation but it does not look to be a big effect – are the changes in distribution of Smo significant different after CCDC66 depletion?

First revision

Author response to reviewers' comments

We thank the reviewers for careful reading of the manuscript and his/her supporting comments and suggestions to further improve the quality of our manuscript and strengthen our conclusions. We are encouraged to see that the reviewers acknowledged the contribution of our results to our understanding of cilium assembly and length control.
We have made all efforts to address the comments and suggestions and detailed our replies in the point-by-point response uploaded with the manuscript files. We highlighted the major changes in the revised manuscript text with "gray".

Reviewer # 1

Advance Summary and Potential Significance to Field:
In this manuscript the authors reported CCDC66 as a new ciliary axoneme/tip protein and investigated its roles in cilium ciliogenesis and function regulation, most significantly, the cilium length control. This paper to some extent provides insights into the mechanism by which ciliary MAPs cooperate to regulate the cilia structure and function

Reviewer 1 Comments for the Author:
I have some concerns or suggestions as followed:

1. When observing the localization of CCDC66 in vivo (for example, Figure 1A), the authors showed only GFP tagged CCDC66, but no GFP only control, how could exclude the possibility that the localization of GFP-CCDC66 in vivo is artefact caused by GFP itself?

We thank the reviewer for pointing this out. Over the years, we generated multiple cell lines stably expressing GGP and have not observed localization of GFP to the cilia. To show that ciliary localization of CCDC66 is not an artefact of GFP in RPE1 cells, we included immunofluorescence data for RPE1::GFP and RPE1::GFP-CCDC66 stable cells stained for acetylated tubulin (cilia marker) in Figure S1A. While GFP localizes to throughout the cell with enrichment in the nucleus, GFP-CCDC66 localized specifically to the primary cilium as marked by acetylated tubulin.

2. In Figure 1F, the mScarlet-Arl13b was in a smear pattern with some granules, in my opinion, mNG-CCDC66 and mScarlet-Arl13b didn't show significant co-localization until 536 min, but this co-localization was lost in 720 min (no or too weak mScarlet-Arl13b signal?), please explain.

The reviewer raises an important point regarding the time-lapse analysis of CCDC66 localization during ciliogenesis. Upon stable expression of mScarlet-Arl13b, we observed granular localization in addition to the cilia. Given that Arl13b is a small GTPase, this granular localization likely represents the vesicular pool of this protein and a consequence of overexpression.

Even though we could capture many movies for dynamics of mNG-CCDC66 and mScarlet-Arl13b in cells stimulated for cilium disassembly, it was very hard to find enough movies to quantify their dynamics during cilium assembly. This was in part due to the low fraction of proteins expressing both markers and the difficulty in finding cells form initiation of ciliogenesis to its completion during the time of imaging. As the reviewer indicated, the stills from the Movie 1 in Fig. 1F show that mNG-CCDC66-positive axonemal stubs form earlier than Arl13b recruitment. To clarify this point, we revised the text in pg. 7 as follows:

"Following serum starvation, mNG-CCDC66 localized to the growing ciliary axoneme, which was followed by recruitment of mScarlet-ARL13B (Fig. 1F). During cilium assembly, the number of CCDC66-positive centriolar satellites gradually decreased and became less concentrated around the basal body (Fig.1F)."

3. The authors found the microtubule-binding fragment (570-948) was sufficient to localize CCDC66 to primary cilium, was it essential?

To test whether the microtubule-binding CCDC66 (570-948) is essential for the ciliary localization of CCDC66, we aimed to generate RPE1 cells stably expressing mNeon-fusions of various CCDC66 truncations. Although we could generate cells expressing CCDC66 (1-408) and CCDC66 (409-948), we could not generate cells expressing CCDC66 (1-570).

We determined the localization of the two new CCDC66 truncations using these stable lines and included a schematic summarizing our results in Fig. 1E. Like mNG-CCDC66 (570-948), mNG (409-948) localized both to the basal body and the primary cilium (Fig. 1E). However, mNG-CCDC66 (1-408) localized only to the basal body, but not the primary cilium (Fig. 1E). This localization data show that CCDC66 (409-948) is necessary and sufficient for the localization of CCDC66 to the primary cilium. We now included this data in Fig. 1E and revised the text in pg. 7 as follows:

"To test whether this fragment targets CCDC66 to the cilia, we generated RPE1 cell lines stably expressing mNeonGreen (mNG) fusions of full-length CCDC66 and its truncations based on their interaction with microtubules and presence of the CCDC66 domain (408-564 a.a.) (Fig. 1E). mNG fusions of CCDC66, CCDC66 (409-948) and CCDC66 (570-948) localized to the basal body, axoneme and the ciliary tip while mNG-CCDC66 (1-408) only localized to basal body. (Fig. 1E-S1C). Taken together, these data indicate that CCDC66 is stably associated with the axoneme and its C-terminal 409-948 a.a. microtubule-binding fragment is sufficient for the basal body and ciliary localization of CCDC66."

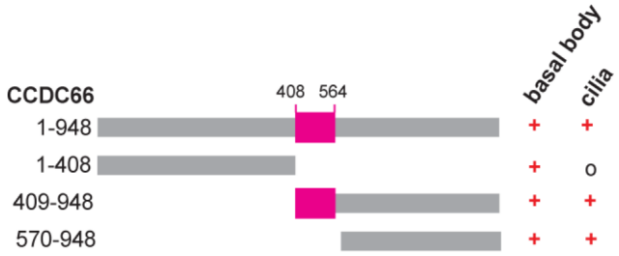


Figure: Schematic representation of localization profiles of mNG-CCDC66 and its fragments.

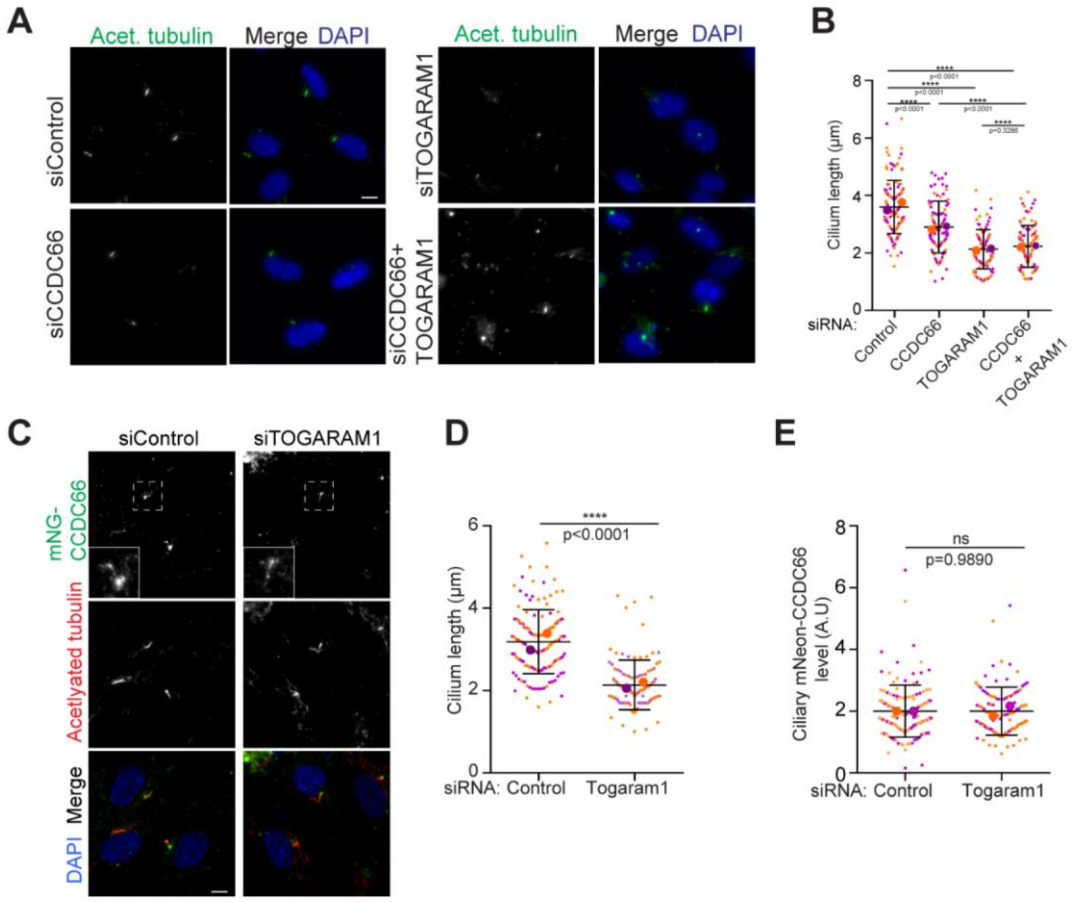
4. There are four axonemal proteins required for cilium length control including CSPP1, ARMC9, CEP104 and TOGARAM1, why only chose CSPP1 and CEP104?

We agree with the reviewer that CCDC66 likely regulates cilium length by cooperating with other proteins in addition to CSPP1 and CEP104. This is supported by the ciliary proximity interactome of CCDC66 we presented in Fig. S4B. Among the proximity interactors of CCDC66, several proteins stand out due to their function in cilium length regulation (i.e. TOGARAM1, ARMC9) as well as link to microtubules and ciliopathies (i.e. CEP162, KIAA0753, MDM1, TTLL5). We have chosen CEP104 and CSPP1 as candidates to investigate mechanisms underlying the ciliary functions of CCDC66 because we showed that they physically interact with CCDC66 in immunoprecipitation experiments (Fig. 5A). While it is definitely worth investigating the relationship of CCDC66 with other MAPs and Joubert-linked proteins such as TOGARAM1 and ARMC9, we think that this is beyond the scope of this paper. We plan to investigate this and other relationships in future studies.

Given the presence of multiple tubulin-binding TOG domains, TOGARAM1 is a prime candidate as a microtubule polymerizer during axonemal elongation (in addition to CEP104). During the revisions, we investigated whether and if so, how CCDC66 cooperates with TOGARAM1 during cilium length regulation using loss-of-function and phenotypic rescue experiments. We summarized and presented the resulting data below.

- Co-depletion of TOGARAM1 and CCDC66 resulted in a more significant decrease than CCDC66 alone and that the phenotype was comparable to that of TOGARAM1 alone. Similar to its relationship to CEP104, this data suggests that CCDC66 might regulate cilium length by acting upon microtubule polymerizers (Figure A- B below).
- Stable expression of mNG-CCDC66 did not rescue the cilium length phenotype associated with TOGARAM1 depletion (Figure C-D below). We could not test whether stable expression of TOGARAM1 rescues the cilium length phenotype of CCDC66 as we were not able to generate RPE1 cells that stably express mNG-TOGARAM1.
- TOGARAM1 depletion did not change the ciliary levels of CCDC66, showing that it is not required for CCDC66 localization to the cilia. (Figure 1C-E below)
- Because TOGARAM1 was not reported to function during cilium assembly, we did not quantify the percentage of ciliated cells upon co-depletion of TOGARAM1 and CCDC66 (Latour et al., 2020).

Taken together, our analysis of the relationship of CCDC66 and TOGARAM1 show that TOGARAM1 does not regulate CCDC66 localization to the cilia and that it functions together with CCDC66 during cilium length regulation. We did not include this set of data to the manuscript as we were not able to perform functional rescue experiments with TOGARAM1 and did not show its physical interaction and co-localization with CCDC66. Therefore, including this data did not fit very well with the flow of the manuscript. If the reviewer suggests that we should include this data, we will revise the manuscript accordingly.



CCDC66 and TOGARAM1 relationship in cilium biogenesis
(A-B) Co-depletions of TOGARAM1 along with CCDC66 results shorter cilia than only CCDC66. (A) RPE1 cells were transfected with two rounds of control, CCDC66, TOGARAM1 and CCDC66 along with TOGARAM1 siRNAs and fixed at 48 hours serum starvation. Following fixation, cells were stained for anti-acetylated tubulin antibody and DAPI. Cilium length was plotted. Data represent the mean ±SD. Magenta and orange represent individual values from two independent experiments.

(100 cilia/experiment, *P < 0.5, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: not significant t-test) Scale bar: 10µm

(C-E) TOGARAM1 does not regulate ciliary CCDC66 levels. (C) RPE1::mNG-CCDC66 cells were transfected with two rounds of control or TOGARAM1 siRNA and serum starved for 48 hours. Following fixation, cells were stained for anti mNeonGreen and Acetylated tubulin antibodies, and DAPI for visualization of DNA. (D) Cilium length was measured and plotted. (E) Ciliary mNG-CCDC66 signal was quantified by measuring the mNeon signal intensity using the area covered by Acetylated tubulin signal. Data represent the mean ±SD. Magenta and orange represent individual values from two independent experiments. (50 cilia/experiment, *P < 0.5, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: not significant t-test) Scale bar: 10µm

5. As a supplement to Figure S4E, the authors should prepare a panel for verifying the siRNA knock down efficiency of CEP104 and CSPP1.

To confirm the knockdown efficiency of CEP104 and CSPP1 by siRNA treatment in RPE1 cells, we blotted extracts from control and depleted cells using antibodies against CEP104, CSPP1 and loading control (alpha-tubulin). The blots in Fig. S4E confirms efficient depletion of CEP104 (104 kDa) and CSPP1 (145 kDa).

6. In CCDC66-depleted cells, Ciliary level of mNG-CEP104 increased (Figure 5E) and expression of mNG-CEP104 compensated for the cilium length defects (Figure 5H), so how to interpret the relationship between increased ciliary localization and rescued cilium length defects? Is there a possibility that the increased ciliary localization of CEP104 promote cilium growth?

The reviewer raises an excellent point on the basis of how CEP104 might rescue the cilium length phenotype associated with CCDC66 depletion. CEP104 was previously described to regulate cilium length via its TOG domain and its TOG domain was shown to increase microtubule polymerization *in vitro* (Yamazoe et al., 2020). Therefore, it is possible that the increased ciliary localization of CEP104 promotes increase in cilia length in CCDC66 depleted cells. We now added the following sentence in the discussion to pg. 17 and 18:

"The cilium length defect of CCDC66-depleted cells is compensated by expression of mNG-CEP104, suggesting that CCDC66 regulates CEP104-mediated axonemal microtubule polymerization. This rescue can be explained by increased CEP104 levels in CCDC66-depleted cells because the TOG domain of CEP104 was shown to promote microtubule polymerization in vitro and rescue the cilium length defect associated with CEP104-depletion (Yamazoe et al., 2020)."

7. According to the IP data, the authors identified that CCDC66 interacted with CEP104 directly, in my opinion, to more straightforwardly support the proposal that 'CCDC66 might be involved in cilium length control via regulation CEP104-mediated axonemal polymerization'(page 15), if possible, it's better to generate a CCDC66 mutant that only disrupt the interaction with CEP104, then see what will happen when transfected to CCDC66-depleted cells.

We agree with the reviewer that disrupting the interaction between CEP104 and CCDC66 via mutants and performing functional rescue experiments are required to directly support our conclusion. To address this, we first aimed to identify the region of CCDC66 that mediates these interactions by performing immunoprecipitation experiments with various CCDC66 truncation mutants. The mutants we used in these experiments were schematized in Fig. 5A. These experiments showed that full length CCDC66 and CCDC66 (409-948) interacted with PCM1, CSPP1, CEP104 and CEP290. CCDC66 (1-408) and CCDC66 (570-948), the two fragments that lack the conserved "CCDC66 domain (408-564 a.a.)", did not interact with these proteins. This result suggests that the conserved CCDC66 domain is required for the ability of CCDC66 to interact with PCM1, CSPP1, CEP104 and CEP290.

Based on the interaction data, we then performed functional rescue experiments to determine the significance of CCDC66-CEP104 interaction during cilium length regulation.

- While stable expression of mNG-CCDC66 (570-948) partially rescued the shorter cilium phenotype in CCDC66-depleted cells, mNG-CCDC66 (409-948) rescued this defect as much as mNG-CCDC66 (Fig. 4C). Given that the latter fragment interacts with CSPP1 and CEP104, we conclude that these interactions and microtubule association contribute to CCDC66 function during cilium length control.
- In contrast to cilium length phenotype, both mNG-CCDC66 (570-948) and mNG-CCDC66 (409-948) partially rescued the defective ciliation phenotype in CCDC66-depleted cells relative to mNG-CCDC66 expression (Fig. 4B). This result indicates that full length protein is required for the activity of CCDC66 during cilium assembly.
- We also performed rescue experiments for defective basal body targeting of CEP290 upon CCDC66 loss using the new cell lines. Reduced centrosomal CEP290 abundance was restored by expression of mNG- CCDC66 (409-948), but not mNG-CCDC66 (570-948) (Fig 4D, 4E). The difference can be explained by the ability of mNG-CCDC66 (409-948) to interact with CEP290 (Fig. 5A).

We included these conclusions to pg. 12 and 14 in the revised manuscript and revised our conclusion on regulation of cilium length directly by CEP104 interaction.

Reviewer # 2

Advance Summary and Potential Significance to Field
CCDC66 is important for ciliogenesis but it localises to many different ciliary associated structures. In this paper, the authors begin to dissect the function of CCDC66 at these different ciliary associated structures by using a set of mutants that have restricted localisations. This has provided important insights into the function of CCDC66 and how it contributes to ciliogenesis.

Reviewer 2 Comments for the Author
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1- Pg6 – the authors state that CCDC66 did not colocalise with CEP290 at the transition zone but based on the images presented there is clearly CCDC66 signal at the transition zone. It is not the region of brightest signal but definitely there and the authors need to acknowledge this. There is an inverse relationship between the CEP290 and CCDC66 signals but still overlap at the transition zone. Moreover, by eye the CCDC66 signal intensity at the centriolar satellites is similar to that at the transition zone.

- We agree with the reviewer that our data in Fig. 1A does not support complete absence of CCDC66 from the transition zone. As the reviewer suggested, our data allows us to conclude on where at the centrosome/cilium complex these proteins are enriched. Therefore, we revised the related text in pg.6 as follows:
"While CEP290 was enriched at the transition zone at the distal end of the centrioles, CCDC66 was enriched at the proximal end of the centrioles and the ciliary axoneme (Fig. S1B)."

- To determine the localization of CCDC66 relative to CEP290 with higher resolution, we performed co- localization experiments using the Zeiss Elyra Lattice SIM microscope. The new immunofluorescence data we present in Fig. S1B supports the enrichment of CEP290 at the transition zone and CCDC66 at the proximal end of centrioles and the ciliary axoneme.

2- Given the drop in CEP290 expression that occurs in the PCM1 KO cells, which the authors acknowledge, I would be a bit more circumspect based on the evidence in concluding the CCDC66-CEP290 interaction is satellite dependent. It is possible that the interaction first occurs there but it

can occur in other places. As the CCDC66 signal overlaps with CEP290 at other points than the satellites. Plus the 'exclusive' localisation of CCDC66 to the centrosome was able to restore CEP290 levels at the transition zone, which suggests CCDC66 can interact with CEP290 at other locations than the centriolar satellites. Does the tethering of CDCC66 to the centrosome alter the localisation of other centriolar satellite proteins? PCM1 looks unaffected but those images are from cells in which endogenous CCDC66 is expressed – on depletion is there a change in localisation?

- We agree with the reviewer regarding the interpretation of the interaction data in terms of where CCDC66 and CEP290 interact. Therefore, we removed the following sentence from pg. 14 where we discuss the interaction data:
  "Consistent with this, CCDC66 co-localized with CEP290 at the centriolar satellites, but not at the transition zone (Fig. 1A)."

  Moreover, we revised the paragraphs in the discussion related to these findings in pgs. 17 and 18 and revised our conclusions on restricted interaction of CCDC66-CEP290 at the satellites as well as exclusion of CCDC66 from the transition zone.

- The reviewer raises an excellent point regarding whether CCDC66-PACT specifically tethers CEP20 to the centrosome or not. To test this, we quantified centrosomal abundance of multiple centriolar satellite proteins.
  , which are CEP63, PCM1, CEP131 and centrin3 (Fig. S3C). Like CEP290, centrosomal levels of CEP63 increased in CCDC66-PACT expressing cells relative to CCDC66-expressing cells. While centrosomal levels of core centriolar satellite proteins CEP131 and PCM1 were reduced upon CCDC66-PACT expression, centrosomal levels of Centrin3 remained unaltered. These results show that CCDC66-PACT expression has variable effects on centrosomal tethering of proteins.

- CCDC66 depletion results in dispersal of centriolar satellites throughout the cytoplasm. We reported this phenotype in the Conkar et al. 2017 study and we include below the related figure form this paper (Conkar et al., 2017).

  https://journals.biologists.com/jcs/article/130/8/1450/56717/The-centriolar-satellite-protein-CCDC66-interacts

Effects of CCDC66 depletion on centriolar satellite organization
RPE1 cells were fixed 72 h after transfection with control siRNA or CCDC66 siRNA, and satellite organization was determined by staining for PCM1 and polyglutamylated tubulin and with DAPI (Conkar et. al., 2017)

Minor points

1- Pg5 - 'although no Joubert-causative CCDC66 mutations are hitherto not reported' – this doesn't make sense. I presume it means no mutations in CCDC66 have been reported?

For clarification, we revised this sentence as follows:
"Early frameshift mutations of CCDC66 in dogs and its deletion in mouse cause retinal degeneration and olfactory deficits (Dekomien et al., 2010; Gerding et al., 2011; Murgiano et al., 2020; Schreiber et al., 2018), and it was also identified as part of a Joubert module, although Joubert-causative CCDC66 mutations have not yet been reported (Latour et al., 2020)."

2- The authors show that the amino acids 570-948 are sufficient to target mNG tothe basal body/cilium but what happens when this region is deleted from CCDC66 –is it necessary for the protein localisation to these regions?

To test whether the microtubule-binding CCDC66 (570-948) is essential for the ciliary localization of CCDC66, we aimed to generate RPE1 cells stably expressing GFP-fusions of various CCDC66 truncations. Although we could generate cells expressing CCDC66 (1-408) and CCDC66 (409-948), we could not generate cells expressing CCDC (1-570).

We determined the localization of the two new CCDC66 truncations using these stable lines and included a schematic summarizing our results in Fig. 1E. Like mNG-CCDC66 (570-948), mNG (409-948) localized both to the basal body and the primary cilium (Fig. 1E). mNG-CCDC66 (1-408) localized to the basal body, but not the primary cilium (Fig. 1E). Taken together, this data show that CCDC66 (409-948) is necessary and sufficient for the localization of CCDC66 to the primary cilium. We now included this data in Fig. 1E and revised the text in pg. 7 as follows:

"To test whether this fragment targets CCDC66 to the cilia, we generated RPE1 cell lines stably expressing mNeonGreen (mNG) fusions of full-length CCDC66, CCDC66 (1-408), CCDC66 (409-948) and CCDC66 (570-948). mNG fusions of CCDC66, CCDC66 (409-948) and CCDC66 (570-948) localized to the basal body, axoneme and the ciliary tip while mNG-CCDC66 (1-408) only localized to basal body. (Fig. 1E). In contrast to mNG-CCDC66, mNG- CCDC66 (570-948) did not localize to centriolar satellites (Fig. S1C). Taken together, these data indiated that CCDC66 is stably associated with the axoneme and its C-terminal 570-948 a.a. microtubule-binding fragment is sufficient for the basal body and primary cilium localization of CCDC66."

Schematic representation of localization patterns of mNG-CCDC66 and fragments.

3- Pg7 – should this be mNG-CCDC66 not GFP-CCDC66 in the second section?

We corrected it.

4- The time lapse examples clearly shows the assembly and disassembly of CCDC66 into the cilium but I was unsure how many movies these conclusions were based on. Can you add this detail into the figure legend. In figure 2D, the mean cilium length for the knockdown cells looks to continue to gradual increase across the time course and not plateau at 200 min. The control rapidly increases in the first 200 mins before levelling out but the knockdown grows slowly throughout.

- We quantified 22 cilia for siControl and 19 cilia movie for siCCDC66 in two independent experiments. We now included this information in the figure legend for Fig. 2D

- We agree with the reviewer that the cilium length did not plateau at 200 min in CCDC66-depleted cells. To clarify this, we revised the related sentence as follows:

  "After the cells committed to cilia formation, cilia grew slower in CCDC66-depleted cells relative to control cells. By 10 h, the average length of cilia in control cells and CCDC66 depleted cells were about 3.2 µm and 2.7 µm, respectively (Fig. 2D)."

4- Pg11 – fwith

We corrected it as "with".

5- S3A – western blot of mNG expression is not very convincing, it is difficult to identify bands in comparison to the parental cells. What are the expected sizes of the fusion proteins?

We performed a new experiment to confirm the expression of the mNG fusion proteins in the stable RPE1 cell lines we generated for the first submission and the revised manuscript. We ran extracts from control cells and cells expressing mNG, mNG-CCDC66, mNG-CCDC66-PACT, mNG-CCDC66 (570-948), mNG-CCDC66 (409-948) and mNG (1-409), and blotted them with primary antibody against mNeonGreen. The blot from this experiment in Fig. S3A confirms the expression of the fusion proteins at the right size, which we highlighted with an arrow in the blot. Moreover, we added information regarding the expected size of each mNG-fusion in the figure legend for Fig. S3A.

We included the following sentence in pg. 11 to explain this data:
"For these experiments, we generated RPE cell lines stably expressing only mNG as a control along with mNG fusions of CCDC66 mutants and validated expression of the fusion proteins by blotting cell extracts with antibodies against mNG and CCDC66 (Fig. S3A)."

6- Pg12 – given the western blot I think it is difficult to make strong conclusions about the expression levels of these constructs. Moreover, given that these cell lines are all generated using similar approaches it is not surprising the localisation of CCDC66 and its mutants are consistent but without the localisation of the endogenous protein this statement about artefacts is difficult to verify. Do the commercial antibodies work for westerns? This might help to give a handle of relative expression levels of the tagged protein and the endogenous protein.

Using the same extracts from the experiment we detailed in point # 5, we performed a new western blot using an antibody against the 355-449 a.a. region of CCDC66 (Sigma, HPA044185). In agreement with the epitope it was raised against, the antibody recognized mNG-CCDC66, mNG-CCDC66PACT, mNG-CCDC66 (409-948) and mNG- CCDC66 (1-408) fusion proteins, but not mNG-CCDC66 (570-948).

Although this blot confirms expression of CCDC66 fusions, it does not reveal information about their expression levels relative to the endogenous protein. Even though we previously had a batch of CCDC66 antibody that recognized endogenous protein by western blotting (Fig. S2A, 2H), the new batch we ordered does not detect endogenous CCDC66 in the RPE1 cell extracts. We also tried two other commercially available and custom-made CCDC66 antibodies (Bethyl Laboratories A303-339A - antigen: 898-948 a.a; Custom-made = antigen: 570-948 a.a.).

However, we did not have success in detecting endogenous CCDC66. Therefore, we did not comment on the expression levels of the fusions proteins relative to the endogenous CCDC66 in the manuscript.

7- 5A/S4C – on the schematic the CCDC66 region 467-570 is highlighted and in the supplementary figure the mutant 1-570 weakly interacts with PCM1 and CSPP1 while the 1-408 mutant does not. What is significant about this region? It is not mentioned in the manuscript? Where does this construct localise?

The CCDC66 conserved domain between 408-564 a.a. is conserved among CCDC66 homologs (shown in magenta in CCDC66 domain organization in Fig. 1E). In the Conkar et al. 2017 paper, we showed that this fragment by itself localizes throughout the cytoplasm and does not interact with microtubules (Conkar et al., 2017). For the revised manuscript, we performed additional localization, immunoprecipitation and functional rescue experiments with different CCDC66 truncations to define the contribution of this and other domains to CCDC66 functions.

For the revised manuscript, we determined the localization of mNG-CCDC66 (1-408) and mNG-CCDC66 (409-948) in addition to mNG-CCDC66 (570-948) using cell lines stably expressing these fusions. Like mNG-CCDC66 (570-948), mNG (409-948) localized both to the basal body and the primary cilium (Fig. 1E). mNG-CCDC66 (1-408) localized to the basal body, but not the primary cilium (Fig. 1E). Taken together, this data show that CCDC66 (409-948) is necessary and sufficient for the localization of CCDC66 to the primary cilium.

Additionally, we performed immunoprecipitation experiments using these constructs to determine which part of CCDC66 is required for mediating its interaction with CSPP1, CEP104, PCM1 and CEP290. The mutants we used in these experiments were schematized in Fig. S4C. The immunoprecipitation experiments showed that full length CCDC66 and CCDC66 (409-948) interacted with PCM1, CSPP1, CEP104 and CEP290. CCDC66 (1-408) and CCDC66 (570-948), the two fragments that lack the CCDC66 conserved domain in the middle, did not interact with these proteins. This result suggests that the CCDC66 conserved domain is required for the ability of CCDC66 to interact with PCM1, CSPP1, CEP104 and CEP290.

Based on the interaction data, we then performed functional rescue experiments to determine the significance of CCDC66-CEP104 interaction during cilium length regulation.

- While stable expression of mNG-CCDC66 (570-948) partially rescued the shorter cilium phenotype in CCDC66-depleted cells, mNG-CCDC66 (409-948) rescued this defect as much as mNG-CCDC66 (Fig. 4C). Given that the latter fragment interacts with CSPP1 and CEP104, we conclude that these interactions and microtubule association contribute to CCDC66 function during cilium length control.
- In contrast to cilium length phenotype, both mNG-CCDC66 (570-948) and mNG-CCDC66 (409-948) partially rescued the defective ciliation phenotype in CCDC66-depleted cells relative to mNG-CCDC66 expression (Fig. 4B). This result indicates that full length protein is required for the activity of CCDC66 during cilium assembly.
- We also performed rescue experiments for defective basal body targeting of CEP290 upon CCDC66 loss using the new cell lines. Reduced centrosomal CEP290 abundance was restored by expression of mNG- CCDC66 (409-948), but not mNG-CCDC66 (570-948) (Fig 4D, 4E). The difference can be explained by the ability of mNG-CCDC66 (409-948) to interact with CEP290 (Fig. 5A).

We included these conclusions to pg. 12 and 14 in the revised manuscript.

8- Pg14 – In addition ot

We corrected it.

9- In the experiments depleting CCDC66 while expressing mNG-CEP104 etc was there any change in the number ciliated cells or was just the length affected?

For the revised manuscript, we assessed the relative functions of CCDC66, CSPP1 and CEP104 during cilium assembly in addition to the cilium length analysis we performed for the first submission. To this end, we quantified the percentage of ciliated cells upon serum starvation in different conditions. Specifically, we depleted CCDC66 in cells stably expressing mNG fusions of CEP104 and CSPP1 and quantified their ciliation phenotype. CCDC66 depletion in RPE1::mNG-CEP104 and RPE1::mNG-CSPP1 cells resulted in a significant reduction in the percentage of ciliated cells (Fig. S4I, S4J). This result shows that stable expression of CEP104 and CSPP1 does not rescue the ciliogenesis defect associated with CCDC66 depletion.

10- Fig. 5J – the authors say that CEP104 RNAi further reduced cilium length but it's not clear what they mean. Further from what? Are they suggesting some synergistic effect?

In Fig. 5J, we depleted CEP104 in RPE1 cells stably expressing mNG-CCDC66 in order to test whether CCDC66 rescues the cilium length defect associated with CEP104. Since CEP104 depletion resulted in shorter cilia relative to control cells, we concluded that CCDC66 expression does not compensate for loss of CEP104. Using "further" in explaining this phenotype led to a confusion, therefore, we revised the sentence as follows:

"Moreover, CEP104 and CSPP1 depletion reduced cilium length in RPE1::mNG-CCDC66 cells (Fig. 5J)."

11- 6H – there is a reduction in the ciliary tip localisation but it does not look to be a big effect – are the changes in distribution of Smo significant different after CCDC66 depletion?

We performed statistical analysis of the data presented in Fig. 6H, which indicated that the reduction in the percentage of cells with ciliary tip localization was not significant. We now added the p values from the statistical analysis to the legend for Fig. 6H.

References

Conkar, D., Culfa, E., Odabasi, E., Rauniyar, N., Yates, J. R., 3rd and Firat-Karalar, E. N. (2017). Centriolar satellite protein CCDC66 interacts with CEP290 and functions in cilium formation and trafficking. *J Cell Sci*.

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Second decision letter

MS ID#: JOCES/2022/260327

MS TITLE: CCDC66 regulates primary cilium length and signaling competence via multi-site interactions with transition zone and axonemal proteins

AUTHORS: Ezgi Odabasi, Deniz Conkar, Jovana Deretic, Umut Batman, Kari-Anne M. Frikstad, Sebastian Patzke, and Elif Nur Firat-Karalar
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but one raised two minor points that will require amendments to your manuscript. I hope that you will be able to carry these out because I would like to be able to accept your paper.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.
I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors addressed all the points in the revision

Comments for the author

No additional comments

Reviewer 2

Advance summary and potential significance to field

This is a thorough revision of the original manuscript describing the functions of CCDC66 and connecting these to different structures associated with the cilium.
The authors have addressed all my concerns with both additional experiments and changes to the text in the manuscript. There also appear to have addressed the other reviewer's concerns as well.

Comments for the author

pg 14 - after 'but not with CEP104' do the authors mean to refer to S4C not 5A?

In Figure 5A is there a specific reason why the second trunctation construct (1-565) does not have the pink region highlighted?

Second revision

Author response to reviewers' comments

We thank the reviewers for careful reading of the manuscript and agreeing that the revision experiments addressed their concerns and strengthened our conclusions. Below is our point-by-point response to the minor points raised by Reviewer 2:

1- pg 14 - after 'but not with CEP104' do the authors mean to refer to S4C not 5A?

We thank the reviewer for pointing to the figure misreferral in pg. 14. We corrected it as "Fig. S4C".

2- In Figure 5A is there a specific reason why the second trunctation construct (1-565) does not have the pink region highlighted?

This actually was not intentional, we forgot to highlight the conserved domain in the (1-564) construct. We revised the domain organization in Fig. 5A to highlight the conserved domain with pink.

Third decision letter

MS ID#: JOCES/2022/260327

MS TITLE: CCDC66 regulates primary cilium length and signaling via interactions with transition zone and axonemal proteins

AUTHORS: Ezgi Odabasi, Deniz Conkar, Jovana Deretic, Umut Batman, Kari-Anne M. Frikstad, Sebastian Patzke, and Elif Nur Firat-Karalar
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.