The FOXJ1 target *Cfap206* is required for sperm motility, mucociliary clearance of the airways and brain development

Anja Beckers, Christian Adis, Karin Schuster-Gossler, Lena Tveriakhina, Tim Ott, Franziska Fuhl, Jan Hegermann, Karsten Boldt, Katrin Serth, Ev Rachev, Leonie Alten, Elisabeth Kremmer, Marius Ueffing, Martin Blum and Achim Gossler
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Original submission

First decision letter

MS ID#: DEVELOP/2020/188052

MS TITLE: The FOXJ1 target Cfap206 is required for sperm motility, mucociliary clearance of the airways and brain development

AUTHORS: Anja Beckers, Christian Adis, Karin Schuster-Gossler, Lena Tveriakhina, Tim Ott, Franziska Fuhl, Jan Hegermann, Karsten Boldt, Katrin Serth, Ev Rachev, Leonie Alten, Elisabeth Kremmer, Marius Ueffing, Martin Blum, and Achim Gossler

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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed.

As you will see the reviewers ask only for changes to the text, and no additional experiments will be required. Just the same, do please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Beckers et al. represents a thorough descriptive and functional analysis of the ciliary protein CFAP206 in both Xenopus and mouse. CFAP206 was identified as a downstream target of FoxJ1 transcriptional regulation which was validated by co-expression studies, FoxJ1 overexpression studies and FoxJ1 loss of function analysis. CFAP206 was found to localize at the

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base of cilia and along the axoneme. Functional analysis of crispr mutants in Xenopus revealed an increase in ciliary beat frequency and a decrease in fluid flow velocity. In mouse ependyma and lung ciliary beat frequency was also increased although no change in flow was observed. In contrast sperm motility was significantly decreased and axonemal structural defects were observed. In general, the experiments are detailed, well controlled and well presented. While cilia have been well studied over the past decade there remains many proteins that have not been functionally analyzed. This paper adds significantly to the field in that it provides a detailed description of a poorly characterized protein as well as helping to describe a novel class of PCD genes that have variant phenotypes.

Comments for the author

Comments:
While in general the discussion is well written I feel that there is still something lacking in the characterization of ciliary flow. What do the authors propose as the reason that flow decreases (or stays the same) while beat frequency increases. This seems counter intuitive unless there is a corresponding decrease in some other aspect of ciliary flow. Ideas that come to mind are ciliary polarity or metachronal synchrony, both of which could be affected by the change in beat frequency. Characterization of this would be ideal but at least a discussion of possible explanation for this contradictory observation would be beneficial.

Related to this is the concern that mice are showing defects associated with loss of ciliary flow despite having no defect in ciliary flow. Hydrocephalus in the brain and mucus build up in the nasal cavity are associated with the loss of flow and yet flow is normal. While I appreciate that the data is the data, a more detailed attempt at explaining these observations would be helpful. For example the authors state that “indicating that most likely aqueduct obstruction and not altered postnatal CGF contributes to the observed early onset hydrocephalus”. It is not entirely clear to me the basis of this statement. What exactly is the evidence for obstruction? This should be described in more detail but even with this detail I am not sure how one gets to that obstruction if flow is normal. Similarly, the accumulation of mucus is easy to explain with a decrease in flow but if flow is normal what do the authors propose as the cause of this accumulation. I feel that this overarching disconnect needs more attention.

I struggled a little bit to understand the localization of CFAP206 in Xenopus. In relationship to both centrin and tubg1 the CFAP206 appears below the basal body. I would interpret this as potentially localizing to the rootlet or at the junction between the basal body and the rootlet. As rootlets have been associated with both polarity and metachronal synchrony this could potentially help explain the discrepancy between beat frequency and flow.

<u>Reviewer 2</u>

Advance summary and potential significance to field

The manuscript by Beckers et al is very careful, well-crafted and clearly argued analysis of the functional metazoan requirement for a novel FOXJ1 target gene, CFAP206. It complements beautifully previous work in Tetrahymena and Chlamydomonas that suggests that CFAP206 is part of the nexin-dynein regulatory complex which is involved in providing mechanical stability to the central pair and radial spokes. This work supports an evolutionarily conserved function for CFAP206 in regulating cilia waveforms necessary to power effective fluid flow patterns in vivo. It extends on functional diversification of the mammalian cilia repertoire in terms of requirement for different cilia proteins, like CFAP206. Whilst immotility and profound ultrastructure defects were observed in mouse mutant sperm other tissues displayed phenotypes suggestive of aberrant cilia beat frequency and/or waveform such as impaired mucociliary clearance and hydrocephaly yet exhibited only subtle yet significant alterations in motility. This paper highlights the very important point about the huge complexity of players involved in building and regulating metazoan cilia motility, which is of fundamental importance to development and disease. With some minor edits and clarifications, I would highly recommend publication in Development, where it will be of great interest across many disciplines including modelling human disease, cilia and biomechanics.

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Comments for the author

Major points:
1. FOXJ1 target versus broader expression: From my understanding, this gene was identified by authors as a FOXJ1 target gene through their multi-organism studies into co-expression as part of a broader pipeline. Whilst the mouse phenotype is clearly a motile cilia phenotype, the narrative is a little complicated by highlighting the non-motile cilia expression by mRNA (Fig 1B, Cf,f', 3A) and protein (Fig 3B,C in mouse kidney collecting duct and subcutaneous connective tissue cell lines- neither of which are motile- see more below). Does the retinal expression disappear in Foxj1 mutants- if not it suggest that there are additional regulators of Cfap206 expression upon which FOXJ1 acts to amplify expression in motile ciliated cell types?
Do the mutants show retinal phenotypes? The problem with this segue in the narrative is that it seems to detract from the main message, without really adding much. Consider streamlining text and emphasis. For example, line 149-151, seems less definitive/true given these issues- clarify the narrative.

2. Specificity of antibodies: More concerning the bands shown in the Figure 3B,C seem not to disappear in the mutants- thus non-specific (Figure 5B), although this is never highlighted for the cell lines that came before. The same specificity 'control' was never shown for the immunofluorescence- does the centrosomal staining (like shown for IMCD3 cells), disappear from the collecting duct cells in Cfap206 mutants? Rabbit antibodies are notorious for sticking to centrosomes… I personally think the cell line work here does not add to the paper but detracts- consider taking it out. Similarly, there is a 'data dump' of CFAP206 interactome in Table S11 without any key details given anywhere about: (1) how the experiment was done; (2) how many samples (biological replicates); (3) how the analysis was done. Most concerningly to me is that the bait protein here CFAP206 which should be the most highly and most significantly changed protein on the list between a null and wild type proteome, is not even on the list. Please take this out- it adds nothing, as it stands and is not introduced, and then only cursorily, in the discussion. Please specify in all figures which antibody was used for blots, IF etc.

Minor points:
1. Introduction, line 54- add '… found on the surface of many…'
2. Introduction, line 84, missing reference/discussion point. The Omran lab has recently published gain-of-function FOXJ1 mutations in PCD patients- Wallmeier et al 2019 (https://doi.org/10.1016/j.ajhg.2019.09.022).
3. Results, line 114, provide reference sequence for gene, transcripts and proteins (i.e. Ensembl build).
4. Results, line 118, clarify. '… (Table S1) which outside a 280aa unique domain of unknown function, with conserved motifs GFC and GIL, lacks other motifs or domains.'
5. Results, line 121, clarify. Does the ISH probe detect both isoforms?
Consider highlighting where on Figure 1A it would bind to?
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9. Results, lines 250-256- spell out wild type.
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11. Results section- please add in reference to Supplementary tables throughout the results section or figure legends as applicable. For example line 289- sperm defects.
12. Discussion, line 313, clarify. 'Several possible mechanisms could underlie these differences.'
13. Discussion, line 321, clarify. 'Indeed ciliary phenotypes in morphants, including cysts and hydrocephalus, seem to support such reasoning, however we were unable to successfully rescue these phenotypes to support specificity.'
14. Discussion, line 326, clarify. '…requisite for some cases of compensation in zebrafish..'. Also an earlier paper (Hall et al 2013: https://doi.org/10.1371/journal.pgen.1003928) had shown CEP131/AZI1 was required for ciliogenesis by depletion, but KOs (nulls) had somehow compensated for the loss, it was not through transcript levels. Males had profound sperm defects suggesting differences in cell type sensitivity to loss, in terms of compensation mechanism, whatever it may be.

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15. Discussion, final paragraph, clarity. Consider also describing that human mutations of CFAP206 may also present in male infertility clinics, or as milder forms of PCD, without clear ultrastructure defects and subtle changes by high speed videomicroscopy complicating diagnosis of this possible cohort.
16. Methods, line 506: add in reference protein identifying used for mapping epitopes.
17. Figure legends, line 842, clarify. Consider adding in a cartoon summary of localization from Xenopus studies to show localition in motile cilia, for non-expert readers, labelling structures in space.
18. Figure 8, quite difficult to see details- consider zoomed views and or reorienting panels to increase size.
19. Figure S1- does staining disappear in mutant- specificity?
20. Table S7- specify which cells/tissue CBF came from.
21. Final recommendation- accessibility. As most of the panels have only two dyes/fluorophores- I would strongly urge the authors to consider a colour blind palette for all immunofluorescence panels in the paper. (magenta/green, cyan/orange- see - https://imagej.nih.gov/ij/docs/guide/146-9.html- about 10% of your male audience may be colourblind).

First revision

Author response to reviewers' comments

We thank both reviewers for their support and constructive comments. We have followed their suggestions as much as possible, as detailed below.

Reviewer 1 Advance Summary and Potential Significance to Field:

The manuscript by Beckers et al. represents a thorough descriptive and functional analysis of the ciliary protein CFAP206 in both Xenopus and mouse. CFAP206 was identified as a downstream target of FoxJ1 transcriptional regulation which was validated by co-expression studies, FoxJ1 overexpression studies and FoxJ1 loss of function analysis. CFAP206 was found to localize at the base of cilia and along the axoneme. Functional analysis of crispr mutants in Xenopus revealed an increase in ciliary beat frequency and a decrease in fluid flow velocity. In mouse ependyma and lung ciliary beat frequency was also increased although no change in flow was observed. In contrast sperm motility was significantly decreased and axonemal structural defects were observed. In general, the experiments are detailed, well controlled and well presented. While cilia have been well studied over the past decade there remains many proteins that have not been functionally analyzed. This paper adds significantly to the field in that it provides a detailed description of a poorly characterized protein as well as helping to describe a novel class of PCD genes that have variant phenotypes

Reviewer 1 Comments for the Author:

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early onset hydrocephalus". It is not entirely clear to me the basis of this statement. What exactly is the evidence for obstruction?
This should be described in more detail but even with this detail I am not sure how one gets to that obstruction if flow is normal. Similarly, the accumulation of mucus is easy to explain with a decrease in flow but if flow is normal what do the authors propose as the cause of this accumulation. I feel that this overarching disconnect needs more attention.

As the referee rightly points out - the data is the data. We agree that what we describe is counter-intuitive and warrants deeper discussions. The evidence that we indeed encounter obstruction in knockout mice is derived from mid-sagittal histological sections of a total of three mutant brains at P6, i.e. every single one that we analysed at that stage (cf. Fig. 6C). At P6, lateral wall-derived flow just sets in.
In order to discuss this issue in a more sensible way, we have deleted the statement of obstructed ventricles from the result part, but added an extended discussion (lines 383-407) instead. This part now reads:

"While loss of CFAP206 clearly affects ciliary beating in respiratory epithelium it remains to be seen how this relates to mucus accumulation. It appears plausible, however, that mucus accumulation is causatively linked to the altered ciliary beat frequency. Altered frequencies may result in subtle alterations of the ciliary wave form and/or changes of beating asymmetry, which might reduce efficient flow in the native lung and lead to mucus accumulation over time.

Similar to flow generated by MCCs of the trachea, ependymal flow in explants of postnatal lateral ventricles (P7) was apparently unaltered, although enlarged ventricles were present accompanied by obstruction of the aqueduct (Fig. 6C). It is unclear whether apparently normal flow is maintained in older animals (which could not be analysed due to restrictions by animal welfare regulations). Aqueduct obstruction, maintenance of flow generated by ependymal cells of the lateral walls of the lateral ventricle and build-up of pressure might all contribute to the highly penetrant progression of ventricle enlargement after P14. The basis for the development of hydrocephalus in *Cfap206* mutants already on P1, prior to the presence of motile cilia on the lateral ventricular walls (Banizs et al., 2005) and the onset of postnatal flow, is less clear. It implies that CFAP206 function is required early-on, already during embryonic development. Consistent with this notion *Cfap206* was detected already at E16.5 in the developing brain, although at low levels (Fig. S6). Analyses of *Ccdc39* mutant mice revealed that ependymal MCCs with motile cilia are present on the ventro-medial wall of the lateral ventricle around P1. Functional impairment of these motile cilia led to enlarged ventricles shortly after birth (Abdelhamed et al., 2018), prior to the emergence of motile cilia on the lateral ventricular walls (Banizs et al., 2005) and the onset of postnatal flow. Loss of CFAP206 might therefore impact on early flow and lead to early aqueduct obstruction, which blocks drainage of cerebrospinal fluid causing subsequent progressive lateral ventricle enlargement. A requirement of ependymal flow to keep the aqueduct postnatally open has been established in *Mdnah5* mutant mice. These lacked directed ependymal flow at lateral ventricles and developed hydrocephalus beginning at P6, with subsequent stenosis of the aqueduct at P12, which was attributed to the absence of postnatal ependymal flow (Ibañez-Tallon et al., 2004)."

I struggled a little bit to understand the localization of CFAP206 in Xenopus. In relationship to both centrin and tubg1 the CFAP206 appears below the basal body. I would interpret this as potentially localizing to the rootlet or at the junction between the basal body and the rootlet. As rootlets have been associated with both polarity and metachronal synchrony this could potentially help explain the discrepancy between beat frequency and flow.

We agree and thank the referee for this valuable hint. We have added this interpretation and changed the description of CFAP206 protein localisation (lines 178-191) as follows.
"GFP-CFAP206 partially overlapped with RFP-tagged centrin4 (Cetn4-RFP) (Cetn4; Zhang and Mitchell, 2016)at basal bodies of epidermal MCCs (Fig. 3Ca). The orthogonal projection depicted in Fig. 3Ca' revealed a defined succession of domains, with proximal (i.e. closest to the axoneme) centrin4-staining, followed by a small zone of centrin4-CFAP206 overlap and a distal CFAP206 domain. Basal body localisation was confirmed by co-staining of GFP-CFAP206

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with the basal foot marker tubulin γ1 (Tubg1; Fig. 3Cb). GFP-CFAP206 partially overlapped with Tubg1, both in the plane parallel to the cell surface (Fig. 3Cb') as well as in an orthogonal projection (Fig. 3Cb''), where GFP-CFAP206 extended towards the distal end of the basal body, at the level of the sub-apical actin network (Fig. 3Cb''). The GFP-CFAP206 domain, therefore, appears below the basal body in relationship to both centrin4 and Tubg1, potentially localising to the rootlet or at the junction between the basal body and the rootlet. GFP-CFAP206 was also expressed throughout the axoneme, though at a lower level, as demonstrated by co-staining with an antibody against acetylated α-tubulin (ac. Tuba4a; Fig. 3Cc,c',c'', d)."

Reviewer 2 Advance Summary and Potential Significance to Field

The manuscript by Beckers et al is very careful, well-crafted and clearly argued analysis of the functional metazoan requirement for a novel FOXJ1 target gene, CFAP206. It complements beautifully previous work in Tetrahymena and Chlamydomonas that suggests that CFAP206 is part of the nexin-dynein regulatory complex which is involved in providing mechanical stability to the central pair and radial spokes. This work supports an evolutionarily conserved function for CFAP206 in regulating cilia waveforms necessary to power effective fluid flow patterns in vivo. It extends on functional diversification of the mammalian cilia repertoire in terms of requirement for different cilia proteins, like CFAP206. Whilst immotility and profound ultrastructure defects were observed in mouse mutant sperm, other tissues displayed phenotypes suggestive of aberrant cilia beat frequency and/or waveform such as impaired mucociliary clearance and hydrocephaly yet exhibited only subtle yet significant alterations in motility. This paper highlights the very important point about the huge complexity of players involved in building and regulating metazoan cilia motility, which is of fundamental importance to development and disease. With some minor edits and clarifications, I would highly recommend publication in Development, where it will be of great interest across many disciplines including modelling human disease, cilia and biomechanics

Reviewer 2 Comments for the Author:

Major points:

1. FOXJ1 target versus broader expression: From my understanding, this gene was identified by authors as a FOXJ1 target gene through their multi-organism studies into co-expression as part of a broader pipeline. Whilst the mouse phenotype is clearly a motile cilia phenotype, the narrative is a little complicated by highlighting the non-motile cilia expression by mRNA (Fig 1B, Cf,f', 3A) and protein (Fig 3B,C in mouse kidney collecting duct and subcutaneous connective tissue cell lines- neither of which are motile-see more below). Does the retinal expression disappear in Foxj1 mutants- if not it suggests that there are additional regulators of Cfap206 expression, upon which FOXJ1 acts to amplify expression in motile ciliated cell types? Do the mutants show retinal phenotypes? The problem with this segue in the narrative is that it seems to detract from the main message, without really adding much. Consider streamlining text and emphasis. For example, line 149-151, seems less definitive/true given these issues- clarify the narrative.

We have followed the suggestion of the reviewer and no longer highlight *Cfap206* non-motile cilia expression in the main text and figures. We just mention this briefly in the text (line 128) and refer to a new supplemental figure (Fig. S1) showing expression in the eye and cell lines. We would like to make these data available to the reader for completeness.

We have not analysed expression in *Foxj1* mutant retinas and have changed the sentence concerning the regulation of *Cfap206* by FOXJ1 (lines 148-150) to read:
"Together, these experiments in mouse and frog demonstrate that FOXJ1 is the decisive transcription factor for *cfap206* activation in cells <u>carrying motile cilia</u> during embryonic development and likely for its expression in adult tissues as well."

2. Specificity of antibodies: More concerning the bands shown in the Figure 3B,C seem not to disappear in the mutants- thus non-specific (Figure 5B), although this is never highlighted for the cell lines that came before. The same specificity 'control' was never shown for the immunofluorescence-does the centrosomal staining (like shown for IMCD3 cells), disappear

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from the collecting duct cells in Cfap206 mutants? Rabbit antibodies are notorious for sticking to centrosomes. I personally think the cell line work here does not add to the paper but detracts- consider taking it out.

The bands shown in the former Fig. 3B show endogenous expression in these cell lines; there are no equivalent mutant cell lines as controls. a-pepI (but not a-pepII) antibodies give rise to background bands in testis lysates, in addition to the specific bands that are absent in mutant testis lysates (marked by arrows in Fig. 5B). We have marked the background bands in Fig. S10 by black and grey asterisks, which shows the full-size Western blots.

We are aware that rabbit antibodies can label centrosomes non-specifically. However, we consider this localisation to be specific, because the GFP-labelled mouse protein also localises to basal bodies in *Xenopus* MCCs.

Similarly, there is a 'data dump' of CFAP206 interactome in Table S11 without any key details given anywhere about: (1) how the experiment was done; (2) how many samples (biological replicates); (3) how the analysis was done. Most concerningly to me is that the bait protein here CFAP206 which should be the most highly and most significantly changed protein on the list between a null and wild type proteome, is not even on the list. Please take this out- it adds nothing, as it stands and is not introduced, and then only cursorily, in the discussion. Please specify in all figures which antibody was used for blots, IF etc.

We apologise for not having provided experimental details with the original submission. CFAP206 is in fact included in the table: it is labelled c6orf165, enriched about 2000-fold in the wild type proteome and detected with 39 peptides and 65% sequence coverage, demonstrating the quality of our mass spectrometric analyses. We have labelled CFAP206/c6orf165 now in Table S11 in yellow, state that c6orf165 is synonymous with CFAP206, and we have added sections in Materials and Methods (lines 587-618) describing experimental details, number of samples/biological replicates and analysis. Given the high quality of the mass spectrometry data we think that this information is valuable for colleagues working in the field, which is why we prefer to retain the data in the manuscript.

Minor points:

1. Introduction, line 54- add '… found on the surface of many…'
We have changed this sentence as suggested (line 51).

2. Introduction, line 84, missing reference/discussion point. The Omran lab has recently published gain-of-function FOXJ1 mutations in PCD patients –Wallmeier et al 2019 (https://doi.org/10.1016/j.ajhg.2019.09.022). –

We have added this information and the reference. The sentence (lines 81-83) now reads: "Nonsense or frameshift mutations in FOXJ1 that cause an autosomal dominant form of PCD have been identified (Wallmeier et al., 2019), and dysfunction of direct or indirect FOXJ1 targets are implicated in the development of numerous human PCD cases (Mukherjee et al., 2019)."

3. Results, line 114, provide reference sequence for gene, transcripts and proteins (i.e. Ensembl build).

We have added a link to the Ensemble entry for *Cfap206* (line 114).

4. Results, line 118, clarify. '… (Table S1) which outside a 280aa unique domain of unknown function, with conserved motifs GFC and GIL, lacks other motifs or domains.'

We have changed this sentence (lines 118-120) as suggested to:
"CFAP206 is an evolutionary conserved protein (Table S1). It lacks known motifs or domains except for a 280aa unique domain of unknown function with conserved motifs GFC and GIL."

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5. Results, line 121, clarify. Does the ISH probe detect both isoforms? Consider highlighting where on Figure 1A it would bind to?

We have added the information that the probe detects both transcripts (line 123) and indicated the location of the probe in Fig 1A.

6. Results, line 146, clarify. '…induction of ectopic cfap206 transcription…'
We have changed this sentence as suggested (line 145).

7. Results, line 198, awkward. '…were observed, which were not consistently encountered….' –
We have changed this sentence as suggested (line 196).

8. Results, line 229, clarify. ' …showed no obvious gross abnormalities'. –
We have changed this sentence as suggested (line 227).

9. Results, lines 250-256- spell out wild type.
We have spelled out wild type throughout the text.

10. Results, line 254, clarify- did you look at waveform? These were disturbed in protists studies.
We were not able to analyse ciliary waveform in our ventricle explants and have added this information (line 255) "…revealed no significant alterations of CGF in mutants compared to wild type in this assay (Fig. 6Fc; Movie 5; Fig. S11; Table S7), and changes of the waveform could not be assessed."

11. Results section- please add in reference to Supplementary tables throughout the results section or figure legends as applicable. For example, line 289- sperm defects.

We have included references to Supplementary tables and figures.

12. Discussion, line 313, clarify. 'Several possible mechanisms could underlie these differences.'
We have changed this sentence as suggested (line 313-214).

13. Discussion, line 321, clarify. 'Indeed ciliary phenotypes in morphants, including cysts and hydrocephalus, seem to support such reasoning, however we were unable to successfully rescue these phenotypes to support specificity.'

We have changed this sentence (lines 323-325) to "Ciliary phenotypes in morphants, including cysts and hydrocephalus, seem to support such reasoning, however we were unable to successfully rescue these phenotypes and prove MO-specificity."

14. Discussion, line 326, clarify. '…requisite for some cases of compensation in zebrafish..'. Also an earlier paper (Hall et al 2013: -https://doi.org/10.1371/journal.pgen.1003928) had shown CEP131/AZI1 was required for ciliogenesis by depletion, but KOs (nulls) had somehow compensated for the loss, it was not through transcript levels. Males had profound sperm defects suggesting differences in cell type sensitivity to loss, in terms of compensation mechanism, whatever it may be.

We have changed this sentence and added the reference to Hall et al as follows (lines 327-329):
"…nonsense-mediated mRNA decay has been shown in several cases to be a pre-requisite for compensation in zebrafish (El-Brolosy et al., 2019), which might be a cell type-specific phenomenon in mice (Hall et al., 2013)."

15. Discussion, final paragraph, clarity. Consider also describing that human mutations of CFAP206 may also present in male infertility clinics, or as milder forms of PCD, without clear ultrastructure defects and subtle changes by high speed videomicroscopy complicating diagnosis of this possible cohort.

We have included these considerations in the final paragraph of the discussion (lines 411-416), which now reads:

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"In conclusion, our descriptive and functional analysis of *Cfap206* in mouse and *Xenopus* demonstrated that this highly conserved ciliary gene functions in defined ciliary contexts, predominantly at post-embryonic stages in both species. Male sterility caused by severe flagellar malformations in mice suggests that mutations of CFAP206 may also underlie male infertility in humans. Mutant alleles might lead to milder forms of PCD, without clear ultrastructure cilia defects and only subtle changes of ciliary movement, which might complicate diagnosis of patients."

16. Methods, line 506: add in reference protein identifying used for mapping epitopes.

We have added criteria used to determine peptides for immunisation in the Materials and Methods section (lines 522-525).

17. Figure legends, line 842, clarify. Consider adding in a cartoon summary of localization from Xenopus studies to show localition in motile cilia, for non-expert readers, labelling structures in space.

We have added a cartoon as suggested (Fig. 3Cd).

18. Figure 8, quite difficult to see details- consider zoomed views and or reorienting panels to increase size.

We have reoriented and rearranged the panels to increase the size.

19. Figure S1- does staining disappear in mutant - specificity?

We have added panels of representative mutant testis sections that clearly show that staining disappears in mutants (now Fig. S2), as was/is also shown in Fig. 5Ch,i.

20. Table S7- specify which cells/tissue CBF came from.

We have indicated that the flow was measured in tracheal explants.

21. Final recommendation- accessibility. As most of the panels have only two dyes/fluorophores- I would strongly urge the authors to consider a colour blind palette for all immunofluorescence panels in the paper. (magenta/green, cyan/orange- see
- https://imagej.nih.gov/ij/docs/guide/146- -9.html- about 10% of your male audience may be colour blind).

We have changed the colours to magenta/green as suggested by the reviewer and added a paragraph to Materials and Methods (lines 548-551) describing the colour manipulation in ImageJ.

Second decision letter

MS ID#: DEVELOP/2020/188052

MS TITLE: The FOXJ1 target Cfap206 is required for sperm motility, mucociliary clearance of the airways and brain development

AUTHORS: Anja Beckers, Christian Adis, Karin Schuster-Gossler, Lena Tveriakhina, Tim Ott, Franziska Fuhl, Jan Hegermann, Karsten Boldt, Katrin Serth, Ev Rachev, Leonie Alten, Elisabeth Kremmer, Marius Ueffing, Martin Blum, and Achim Gossler

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

The overall evaluation is positive and we would like to publish a revised manuscript in Development.

The reviewers did request two very minor additions to the manuscript and I agree these will substantially improve the work. First, please provide specific details as to the genetic background of the mice, if available, as requested by reviewer 1. Second, please submit the result of the proteomic experiments to the PRIDE database.

Reviewer 1

Advance summary and potential significance to field

The revised version represents a significant improvement and addresses my previous concerns.

Comments for the author

In general, I believe the authors have done a reasonable job addressing my concerns and I support publication. However, there is one issue that I should have mentioned previously but failed to, that I would like the authors to at least consider. I do not want to hold up publication but there is one aspect to the mouse studies that I am unclear about and that I think might warrant some discussion. I think it is well appreciated in the field that the inbred mouse lines represent a significant liability to the study of ciliary phenotypes associated with hydrocephalus. This should be stated clearly so that it remains a feature of the collective knowledge. As regards to this work, there is not much information in the methods about the genetic background of the CFAP206 line that was generated. There is one sentence that states “The phenotype of Cfap206Δex4 mice was analysed on a mixed genetic background.” Given the clear limitations to mouse ciliary studies in the standard inbred lines I think this should be described a little better. The inbred lines for unknown reasons (at least to me) are super susceptible to hydrocephaly. I think it would be useful to people in the field to understand how this issue was dealt with (details of the mixed genetic background). If I am wrong about what the authors mean by mixed genetic background (e.g. they are using inbred lines) then this is relevant to the discussion about the phenotype and why they do not see the same things between mouse and frogs.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Beckers et al is very careful, well-crafted and clearly argued analysis of the functional metazoan requirement for a novel FOXJ1 target gene, CFAP206. It complements beautifully previous work in Tetrahymena and Chlamydomonas that suggests that CFAP206 is part of the nexin-dynein regulatory complex which is involved in providing mechanical stability to the central pair and radial spokes. This work supports an evolutionarily conserved function for CFAP206 in regulating cilia waveforms necessary to power effective fluid flow patterns in vivo. It extends on functional diversification of the mammalian cilia repertoire in terms of requirement for different cilia proteins, like CFAP206. Whilst immotility and profound ultrastructure defects were observed in mouse mutant sperm, other tissues displayed phenotypes suggestive of aberrant cilia beat frequency and/or waveform such as impaired mucociliary clearance and hydrocephaly yet exhibited only subtle yet significant alterations in motility. This paper highlights the very important point about the huge complexity of players involved in building and regulating metazoan cilia motility, which is of fundamental importance to development and disease.

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Comments for the author

The authors have made all the changes to and/or clarified any of the minor issues I had in the original submission. As such I would highly recommend publication in Development, where it will be of great interest across many disciplines including modelling human disease, cilia and biomechanics.

I would ask that the authors upload the proteomics dataset to PrideDB and provide the accesssion number prior to publication so that the field can look re-iterrogate interactions or run different analysis software. It is a very valuable ressource that should be open.

Second revision

Author response to reviewers' comments

The requested additional information concerning the genetic background of mutant mice has been included in the Materials and Methods section as follows (lines 440-445):

Germ line deletion of exon 4 was achieved by crossing of Cfap206loxP; ZP3:Cre (de Vries et al., 2000) double heterozygous females to wild type males. The floxed allele was originally generated on the C57BL/6 background. Breeding to FLPe and ZP3:Cre mice generated a mixed genetic background (predominantly 129Sv/CD1), on which the strain was maintained. The phenotype of Cfap206Dex4 mice was analysed on this mixed genetic background.

The information concerning mass spec data accession has been included in the Materials and Methods section as follows (lines 621-623):
The full mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD018554.

Third decision letter

MS ID#: DEVELOP/2020/188052

MS TITLE: The FOXJ1 target Cfap206 is required for sperm motility, mucociliary clearance of the airways and brain development

AUTHORS: Anja Beckers, Christian Adis, Karin Schuster-Gossler, Lena Tveriakhina, Tim Ott, Franziska Fuhl, Jan Hegermann, Karsten Boldt, Katrin Serth, Ev Rachev, Leonie Alten, Elisabeth Kremmer, Marius Ueffing, Martin Blum, and Achim Gossler
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.

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