EMBO reports

Parental centrioles are dispensable for deuterosome formation and function during basal body amplification

Huijie Zhao, Qingxia Chen, Chuyu Fang, Qiongping Huang, Jun Zhou, Xiumin Yan, and Xueliang Zhu

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 6 September 2018

Thank you for the submission of your research manuscript to our journal. I apologize again for the delay in handling your manuscript, but we have only recently received the third referee report. Please find all reports copied below.

As you will see, the referee opinions are divided and referee 2 raises important concerns regarding the novelty and general interest of the reported findings. Upon further discussion of this aspect with the referees, referee 1 and 3 reinforced their view that the demonstration that deuterosomes can form de novo (even if they might normally form in association with parental centrioles) was an important finding. On balance and given the support from at least two referees we have therefore decided to invite you to revise your manuscript for EMBO reports with the understanding that the referee concerns must be fully addressed and their suggestions taken on board.

Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please

follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

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Referee #1:

This manuscript addresses an interesting discrepancy in the literature. The discrepancy has to do with the source of the deuterosome, a centriole-nucleating structure found only in multiciliated cells, and only during early stages of their differentiation. The deuterosome is distinguished by the presence of a protein, deup1, that is only substantially expressed in multiciliated cells, which make more than 100 centrioles during differentiation. The deuterosome had been thought to form freely from components in the cytoplasm of multiciliated cells, but another group published a paper (Al Jord, 2014) showing that the deuterosome is derived from the younger of the two mother centrioles in a multiciliated cell. The implication was that the centriole was required to form the deuterosome.

This manuscript resolves several differences between the original work from this lab and the more recent Al Jord, et al. paper. First, they show that the deuterosome-based centriole formation pathway is similar in two types of multiciliated cells, derived either from the trachea or brain ependymal cells. Second, they show that early-stage deuterosomes exist free of the original two centrioles in differentiating multiciliated cells, suggesting that they are not made proximal to the centrioles. Third, and most importantly, they use RNAi against Plk4 to deplete centrioles from cells prior to initiating differentiation. Plk4 is required for centriole duplication in cycling cells, and eliminating it results in reduction of centriole number by dilution during divisions of the precursor cells. They find that deuterosomes still form in these cells, although centrioles do not, presumably because of a

requirement for Plk4 activity.

The data are of high quality, and make the points claimed in the text. I have only two concerns about the manuscript:

1) The authors use lentivirally-mediated Plk4 RNAi for the critical experiment of eliminating centrioles from cells prior to deuterosome formation. This is a reasonable approach, but should be accompanied by the same experiment, but using centrinone, a small molecule inhibitor, to inhibit Plk4 directly. This is a conceptually simpler experiment and one that would be very valuable to the community.

2) The ultimate conclusion of the paper, on pg. 11, is rather weak. The text states that "Our results, however, do not necessarily contradict with the observation that the young MC can serve as a deuterosome nucleation site." But the point of this manuscript is to test whether the mother centriole (MC) is the site of deuterosome formation. It is clear from the results here that centrioles are not required for deuterosome formation under conditions of Plk4 depletion, but if deuterosomes really do normally form at the younger of the two mother centrioles, then that is really a moot point. It should be possible to make a more definitive statement here, perhaps relying on live cell imaging to make clearer the source of the deuterosomes.

Referee #2:

The simple manuscript by Zhou et al. describes the formation of the deuterosome in both mTECs and mEPCs. In contrast to what has been published from the Spassky group (Al Jord et al.) they find that deuterosomes are capable of nucleating in the absences of parental centrioles. They determine this by treating cultures with shRNA to Plk4 which results in a loss of centriole duplication and following cell division, a dilution of overall centriole number such that some cells have 1 or 0 centrioles rather than 2. In these cells they find close to normal numbers of deuterosomes. This paper also does a nice job of describing the stages of deuterosome formation and centriole amplification in these two cell culture models. Overall the data presented in this manuscript is of good quality and the imaging is very nice. However, the paper is essentially completely descriptive with no mechanistic insight whatsoever. As such I find that publication in a journal the caliber of EMBO is not warranted. Their claim of importance is based on disputing the claims of the Al Jord Nature paper. When I read the Al Jord paper I had serious doubts about their claims of centriole requirement for deuterosome formation. Therefore, I do think that the findings in this paper are relevant and will be an important addition the field in clarifying that misconception. While this paper convincingly shows that centrioles are not required for deuterosome formation, even this finding is not completely novel as this was also the conclusion one would draw fromo the Mori et al. paper (Nat Comm 2017) which found Deup1 aggregates in the nucleus upon expression of a nuclear E2F4 thus indicating that deuterosomes could form in the nucleus away from the parental centrioles. In conclusion while I think this is a nice paper that should be published, I do not think that it is even close to the level of EMBO. Unless something beyond the purely descriptive data that is currently presented can be added, I think this paper would be better suited for a more specialized journal.

Comments:
While I think one of the strengths of this paper is the use of cell culture models, at least some discussion should be aimed at the possibility that the in vivo situation, or cultured tissue situation (as in Al Jord) could be different. The fact they are using a slightly different systems does leave open the possibility that the parental centrioles are still required in vivo or in explanted tissue.

It is standard in the centriole field to call the older of the two centrioles "the mother" and the younger "the daughter" yet throughout this paper they simply refer to both as "mothers". While I understand that in the context of mTECs centriole amplification they both accumulate "mother" markers and can both act to nucleate "daughter" centrioles, that does not change the fact that one of them is in fact older. This was at the heart of the Al Jord paper since in the premature cells only the younger of the parental centrioles (the daughter) was claimed to be required for deuterosome formation. I find the use of mothers for both centrioles confusing and contrary to the field. I think the authors should try to keep this distinction clear. This is particularly confusing when the authors perform the Plk4 shRNA analysis since they refer to all mothers as "old" when some would be older

than others. The important distinction, which the authors make is that the centrioles have now gone through a cell cycle and have accumulated "mother" markers like odf2.

Related to above...The authors state: "To confirm that the 1MC-cells indeed contained only the old MC.... Thus, this remaining MC is indeed the old MC." This statement implies that of the two original MCs this is "the old" one. The original cell had a mother and a daughter and thus I read this to sound like the 1MC cells all contain the original mother which is not true. 50% of them contain the original daughter or the young MC but these have now acquired the mother markers. In fact, both "the old and the new" one have now gone through a cell cycle and have been licensed to become mothers. I believe the authors know what they are talking about I just feel that it is confusing to the reader. Some effort to simplify the terminology would be beneficial.

"The cilium became elongated at SS d3 (Fig. 4c)." While this looks true based on the images provided cilia have a lot of variation and to include this statement some quantification should be included.

It is a bit odd that centrin-GFP strongly localizes to the base of the cilia. More concerning is that 4c shows that the big centrin-GFP lines are not cilia (no acetylated tubulin), and they don't stain with centriole markers so what are they and if you are getting random blobs of centrin-GFP is it a good marker for other experiments? Sometimes it is used as a centriole marker, sometimes a cilia marker and sometimes a nothing marker. That said I guess it is a reasonable marker for infection efficiency. However, there is never co-staining for daughter centriole markers so it is hard to justify this as a marker for pan-centrioles.

Figure 3d (0 MC). There still appears to be Cep63 foci. Please Explain.

References...Given how few papers there are published on deuterosomes the authors should be more inclusive to the field. Mori et al. stands out as a clear miss, while Mori et al. is referenced for a technical reason, their data actually strongly supports this paper and it seems like making a connection with that study would strengthen this paper. Also to mention "hyperactivated through overexpression of its key regulators" but not reference any of the numerous papers that have worked out the transcriptional mechanisms driving this overexpression seems inappropriate. Other examples of important papers that have been left out are also present and a more thorough effort should be made.

Referee #3:

Centrioles perform a dual function in eukaryotic cells, forming centrosomes in dividing cells and cilia in non-dividing cells. While many interphase cells possess a solitary cilium, nucleated by the older, mother centriole, certain terminally differentiated cells assemble up to several hundred motile cilia, whose coordinated beating helps generate fluid flow in the human respiratory tract, brain and oviduct. Assembly of these multiple cilia requires a fundamental change from the usual 'once and only once' mode of replication to generate the requisite number of centrioles. In vertebrates, centriole amplification in multiciliated cells involves both the assembly of multiple daughter centrioles on the same parent and assembly on non-centriolar generative structures called deuterosomes.

Here, Zhao and Chen propose that deuterosomes do not arise from association with the original parental centrioles as previously reported (Al Jord, Nature 2014), but 'de novo'. This certainly is a finding of sufficient interest for the readership of EMBO Reports. As detailed below, the data does not quite support such a sweeping conclusion. Nevertheless, I still support publication of this manuscript subject to a thorough revision of the text and the inclusion of some essential controls.

Major points

1. The authors interpret their results as a falsification of the main finding of Al Jord et al, that deuterosomes form in association with parental centrioles. This is not correct. If one discounts the argument made from the data in Figures 1 and 2 (which to me is ambiguous at best), the finding that deuterosomes can form in the absence of a parental centriole following Plk4 RNAi does not mean

that they normally do so. The same argument could be made for canonical centriole duplication, which usually takes place in association with a parental centriole, but can also occur de novo following centriole removal (eg La Terra, JCB 2005, a literature well worth discussing). The evidence for centriole association of deuterosome formation in Al Jord et al, including live microscopy and EM, is quite strong and not invalidated by anything presented here.

2. Contrary to what the authors state (p4), Al Jord et al do not claim that procentrioles form only when deuterosomes are in the vicinity of a parental centriole or that deuterosomes are already mature (full size) upon release. As shown eg in Figure S6e of that paper, procentriole number increases upon release from the mother centriole and deuterosomes subsequently grow significantly larger. Showing much the same in Figures 1 and 2 and presenting this as evidence against mother centriole-'guided' deuterosome formation is therefore a straw man argument.

3. The strongest argument the authors present for centriole-independent deuterosome formation is their formation in cells lacking parental centrioles following Plk4 RNAi (Figures 3, 4). However, I'm not entirely convinced by the data presented for the absence of parental centrioles. Cep63/Cep164/Odf2 foci, though dim and somewhat too numerous, are visible in all 0MC cells (Figures 3d, 4a, b). Unexplained centrin streaks are also present in the same cells. Without EM, how can the authors be confident that centrioles are indeed not present? At the very least, the authors should present evidence (eg immunofluorescence microscopy with multiple centriolar markers) confirming that their antibodies indeed reliably and specifically detect centrioles.

Other points

4. The manuscript fails to provide an adequate overview of the existing literature on canonical centriole assembly (barely mentioned, but clearly relevant) and deuterosome-mediated centriole assembly. For the latter, at a minimum the following papers need to be cited and fully discussed: Klos-Dehring, Dev Cell 2013 (CCDC78 as a deuterosome protein), Mori, Nat Commun 2017 (E2f4 in de novo formation of deuterosomes), Vladar, JCB 2007 (establishment of mTECs as a model system for studying multiciliogenesis).

Moreover, a sentence like "We have previously found that the MC-mediated centriole amplification still uses the canonical ring-shaped platform around its basolateral wall, which contains the Cep63-Cep152-Plk4 complex and other components (Banterle & Gonczy, 2017, Brown, Marjanovic et al., 2013, Habedanck, Stierhof et al., 2005, Hatch, Kulukian et al., 2010, Nigg & Holland, 2018, Sir, Barr et al., 2011) but is hyperactivated through overexpression of its key regulators (Yan, Zhao et al., 2016, Zhao, Zhu et al., 2013), similar to what have been demonstrated in cycling cells by overexpressing Plk4, Cep152, SAS6, or STIL (Arquint, Sonnen et al., 2012, Dzhindzhev, Yu et al., 2010, Kleylein-Sohn, Westendorf et al., 2007, Strnad, Leidel et al., 2007, Vulprecht, David et al., 2012)." (p3, introduction) is not only overly long, but gives the impression that the authors are solely reponsible for our current understanding of centriole assembly in multiciliated cells. Similarly, "Fully assembled centrioles are eventually released from their "cradles" by APC/C-activated proteolysis and mature into basal bodies (Al Jord, Shihavuddin et al., 2017, Zhao et al., 2013)." (also p3) misleadingly suggests that the authors' 2013 paper contributed to the identification of a role for the APC/C in release of newly formed centrioles.

5. The authors give the impression that Al Jord et al were alone in suggesting deuterosomes arise in association with parental centrioles (p4, introduction) and that this would potentially be a unique feature of mouse ependymal cells. This is not so. For example, Kalnins and Porter (Z Zellforsch 1969) reported a mother centriole association of deuterosomes (here called cylindrical cores) in the chick tracheal epithelium.

6. If deuterosome numbers per cell were assessed 'in cells containing deuterosomes' (legend to Figure 3g), does this not leave the possibility that there were Plk4-depleted cells that did not form any deuterosomes? This should be excluded by separately quantitating the number of cells without deuterosomes. Also, what criterion was used to assess deuterosome number - Deup1, Cep152?

7. The authors only briefly mention that procentriole formation was abolished in Plk4-depleted cells (p9). Since this strengthens their argument that RNAi was effective in eliminating new centriole assembly (also of parental MCs), they may want to present proper quantiation of this. If not read

carefully, the text gives the mistaken impression that Plk4 is not required for deuterosome-mediated centriole assembly (eg concluding sentence of abstract), which is not what the authors are seeking to claim.

Minor comments
8. Labeling of insets in image panels is somewhat idiosyncratic and confusing. For example, in Figure 1c, which inset is showing Deup1, Cep152 and Centrin is only apparent from the multi-color merge. I do appreciate, though, that insets are presented in black and white for best contrast.

9. There seem to be a few instances of incorrect inset placement. Thus, the top set of insets in the middle lower panel of Fig. 4a shows Cep152 not Cep164 based on the merge. The same appears to be the case in the upper middle panel of Figure 4b (Odf2 vs Cep152).

10. Quantifications of deuterosome diameter based on 3D-SIM images are presented with an unlikely number of significant figures (eg 211.1 +/- 59.9 nm, p5). Since measured particle size depends on image thresholding, the methods section needs to be more clear how this was determined.

11. Centrin is an unfortunate, if frequently used, marker for centrioles, since it also localizes to centriolar satellites (Dammermann, JCB 2002; Hori, MBOC 2015). This likely explains the 'aggregates' observed by the authors in mEPCs (p6).

12. 'punctual' (p6) should read 'punctate'.

13. The liberal use of acronyms and unusual abbreviations is making this manuscript unnecessarily difficult to read. It's bad enough to have mother centrioles referred to throughout as MCs. But who remembers that 'SS d2' means 2 days post serum starvation or that Plk4i/Ctrli-expressing mEPCs are Plk4/Control RNAi-depleted cells? MCD and DD (Figure 1a, presumably mother centriole and deuterosome-dependent centriole assembly pathways) are nowhere defined in the paper.

1st Revision - authors' response 8 December 2018

Answers to reviewers' comments:

Referee #1:

This manuscript addresses an interesting discrepancy in the literature. The discrepancy has to do with the source of the deuterosome, a centriole-nucleating structure found only in multiciliated cells, and only during early stages of their differentiation. The deuterosome is distinguished by the presence of a protein, deup1, that is only substantially expressed in multiciliated cells, which make more than 100 centrioles during differentiation. The deuterosome had been thought to form freely from components in the cytoplasm of multiciliated cells, but another group published a paper (Al Jord, 2014) showing that the deuterosome is derived from the younger of the two mother centrioles in a multiciliated cell. The implication was that the centriole was required to form the deuterosome.

This manuscript resolves several differences between the original work from this lab and the more recent Al Jord, et al. paper. First, they show that the deuterosome-based centriole formation pathway is similar in two types of multiciliated cells, derived either from the trachea or brain ependymal cells. Second, they show that early-stage deuterosomes exist free of the original two centrioles in differentiating multiciliated cells, suggesting that they are not made proximal to the centrioles. Third, and most importantly, they use RNAi against Plk4 to deplete centrioles from cells prior to initiating differentiation. Plk4 is required for centriole duplication in cycling cells, and eliminating it results in reduction of centriole number by dilution during divisions of the precursor cells. They find that deuterosomes still form in these cells, although centrioles do not, presumably because of a requirement for Plk4 activity.

The data are of high quality, and make the points claimed in the text. I have only two concerns about the manuscript:

1) The authors use lentivirally-mediated Plk4 RNAi for the critical experiment of eliminating centrioles from cells prior to deuterosome formation. This is a reasonable approach, but should be accompanied by the same experiment, but using centrinone, a small molecule inhibitor, to inhibit Plk4 directly. This is a conceptually simpler experiment and one that would be very valuable to the community.
We thank this reviewer for recognizing the strength and implications of our study. Following the request, we treated the ependymal precursor cells with centrinone and also observed efficient deuterosome assembly in mEPCs with no or one parental centriole. The data are presented in Fig. 6 in the revised manuscript.

2) The ultimate conclusion of the paper, on pg. 11, is rather weak. The text states that "Our results, however, do not necessarily contradict with the observation that the young MC can serve as a deuterosome nucleation site." But the point of this manuscript is to test whether the mother centriole (MC) is the site of deuterosome formation. It is clear from the results here that centrioles are not required for deuterosome formation under conditions of Plk4 depletion, but if deuterosomes really do normally form at the younger of the two mother centrioles, then that is really a moot point. It should be possible to make a more definitive statement here, perhaps relying on live cell imaging to make clearer the source of the deuterosomes.
We thank the reviewer for the comment and this constructive suggestion. In the revised manuscript, we have included the results of live cell imaging using GFP-Deup1 as deuterosome marker (Fig. 3; Movie EV1-2). We observed that deuterosomes emerge from a widely variety of locations in mEPCs, rather than from a certain position or small area implicated in the location of the daughter centriole. They initially appear as tiny dim foci, followed by a gradual increase in both size and GFP fluorescence intensity. Furthermore, several deuterosomes were observed to appear from different locations within minutes or an hour, which is in contrast to the report by Al Jord and colleagues that it takes at least two hours to form a halo at and release it from the daughter centriole. These results strongly suggest that deuterosomes self-assemble efficiently in mEPCs. We have modified the text accordingly.

Referee #2:

The simple manuscript by Zhou et al. describes the formation of the deuterosome in both mTECs and mEPCs. In contrast to what has been published from the Spassky group (Al Jord et al.) they find that deuterosomes are capable of nucleating in the absences of parental centrioles. They determine this by treating cultures with shRNA to Plk4 which results in a loss of centriole duplication and following cell division, a dilution of overall centriole number such that some cells have 1 or 0 centrioles rather than 2. In these cells they find close to normal numbers of deuterosomes. This paper also does a nice job of describing the stages of deuterosome formation and centriole amplification in these two cell culture models. Overall the data presented in this manuscript is of good quality and the imaging is very nice. However, the paper is essentially completely descriptive with no mechanistic insight whatsoever. As such I find that publication in a journal the caliber of EMBO is not warranted. Their claim of importance is based on disputing the claims of the Al Jord Nature paper. When I read the Al Jord paper I had serious doubts about their claims of centriole requirement for deuterosome formation. Therefore, I do think that the findings in this paper are relevant and will be an important addition the field in clarifying that misconception. While this paper convincingly shows that centrioles are not required for deuterosome formation, even this finding is not completely novel as this was also the conclusion one would draw fromo the Mori et al. paper (Nat Comm 2017) which found Deup1 aggregates in the nucleus upon expression of a nuclear E2F4 thus indicating that deuterosomes could form in the nucleus away from the parental centrioles. In conclusion while I think this is a nice paper that should be published, I do not think that it is even close to the level of EMBO. Unless something beyond the purely descriptive data that is currently presented can be added, I think this paper would be better suited for a more specialized journal.

We appreciate that the reviewer recognizes the value of our study. The Mori paper reports the importance of cytoplasmic E2f4 in deuterosome formation but does not address the origin of deuterosomes at all. Furthermore, no attempts are made in the paper to clarify that the Deup1 aggregate in the nucleus was a deuterosome. In the revised manuscript, we have included live cell imaging results to further strengthen the point that deuterosomes assemble spontaneously in mEPCs with intact parental centrioles (Fig. 3). Furthermore, we used the Plk4 inhibitor centrinone to confirm the results of Plk4 RNAi (Fig. 6). We have also extensively revised the manuscript for better clarity. With these improvements, we hope that the reviewer would find the manuscript important to the field and suitable for publication in EMBO Reports.

Comments:

While I think one of the strengths of this paper is the use of cell culture models, at least some discussion should be aimed at the possibility that the in vivo situation, or cultured tissue situation (as in Al Jord) could be different. The fact they are using a slightly different systems does leave open the possibility that the parental centrioles are still required in vivo or in explanted tissue.
Al Jord and colleagues used the same cell differentiation system as we did to address deuterosome formation (please refer to Fig. 1c of their Nature paper).

We agree with the reviewer that studies performed in *in-vitro* system may not exactly reflect the *in-vivo* situation. In the revised manuscript, we have included a sentence to leave this as an open question (the last sentence of the main text).

It is standard in the centriole field to call the older of the two centrioles "the mother" and the younger "the daughter" yet throughout this paper they simply refer to both as "mothers". While I understand that in the context of mTECs centriole amplification they both accumulate "mother" markers and can both act to nucleate "daughter" centrioles, that does not change the fact that one of them is in fact older. This was at the heart of the Al Jord paper since in the premature cells only the younger of the parental centrioles (the daughter) was claimed to be required for deuterosome formation. I find the use of mothers for both centrioles confusing and contrary to the field. I think the authors should try to keep this distinction clear. This is particularly confusing when the authors perform the Plk4 shRNA analysis since they refer to all mothers as "old" when some would be older than others. The important distinction, which the authors make is that the centrioles have now gone through a cell cycle and have accumulated "mother" markers like odf2.
Related to above...The authors state: "To confirm that the 1MC-cells indeed contained only the old MC.... Thus, this remaining MC is indeed the old MC." This statement implies that of the two original MCs this is "the old" one. The original cell had a mother and a daughter and thus I read this to sound like the 1MC cells all contain the original mother which is not true. 50% of them contain the original daughter or the young MC but these have now acquired the mother markers. In fact, both "the old and the new" one have now gone through a cell cycle and have been licensed to become mothers. I believe the authors know what they are talking about I just feel that it is confusing to the reader. Some effort to simplify the terminology would be beneficial.
We apologize for the confusion in the nomenclatures and appreciate the comments. In the revised manuscript, we use "the mother centriole" and "the daughter centriole" when the identity of parental centrioles needs to be described. Otherwise we use "parental centriole(s)" to avoid confusion.

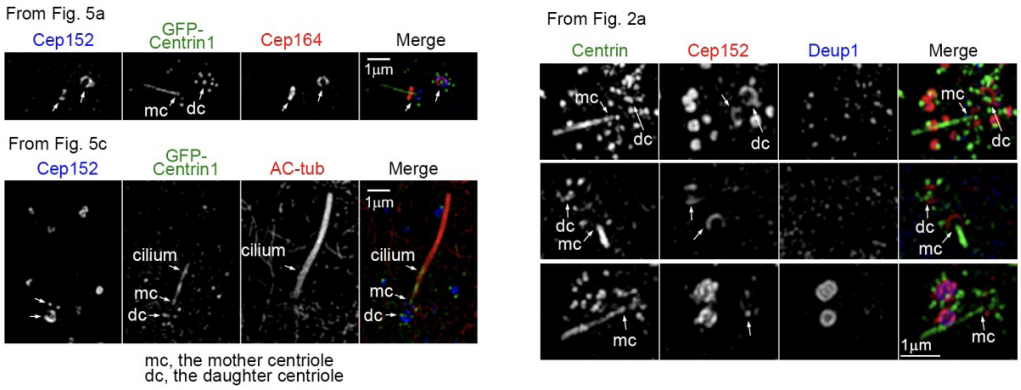
"The cilium became elongated at SS d3 (Fig. 4c)." While this looks true based on the images provided cilia have a lot of variation and to include this statement some quantification should be included.
We have quantified the ciliary length as requested and presented the results in Fig. EV2 in the revised manuscript. The results show that the ciliary length increased from 1.2 ± 0.5 µm at day 0 to 3.9±1.4 µm at day 3.

It is a bit odd that centrin-GFP strongly localizes to the base of the cilia. More concerning is that 4c shows that the big centrin-GFP lines are not cilia (no acetylated tubulin), and they

don't stain with centriole markers so what are they and if you are getting random blobs of centrin-GFP is it a good marker for other experiments? Sometimes it is used as a centriole marker, sometimes a cilia marker and sometimes a nothing marker. That said I guess it is a reasonable marker for infection efficiency. However, there is never co-staining for daughter centriole markers so it is hard to justify this as a marker for pan-centrioles.

We frequently observed that both endogenous Centrin (Fig. 2) and exogenous GFP-Centrin1 (Fig. 4-5) localized to the central region of the ciliary base (above the transition zone), in addition to the typical localization on centrioles (please also refer to Figure I below). Such localization was also observed for Centrin2-GFP by Al Jord and colleagues (please refer to Fig. 3b of their Nature paper). The physiological function of such a localization remain to be elucidated in the future.

Fig. I



In addition, GFP-Centrin1 and endogenous Centrin also tend to form aggregates in differentiating multiciliated cells (please refer to Fig. 4d,f and Fig. 5 in the revised manuscript) (Zhao et al., 2013). Such aggregates, however, do not show costaining with other centriolar markers and can therefore be easily distinguished.

As requested, we costained the virus-infected ependymal progenitor cells (day 0) with the daughter centriole marker Centrobin. The results showed that Centrobin decorated one of the parental centrioles marked by GFP-Centrin1 and Cep152 in control ependymal progenitors (Fig. EV1a), indicating that GFP-Centrin1 can serve as a marker for pan-centrioles (in fact we always use at least two different markers to identify parental centrioles). In the Plk4-depleted progenitors containing one parental centriole, only approximately 8% of the cells contained the daughter centriole (Fig. EV1b). Furthermore, the ring-shaped Centrobin staining was not detected in the Plk4-depleted progenitors with no parental centriole, judged by Cep152 and GFP-Centrin1 (Fig. EV1a). These results are consistent with those obtained with the mother centriole markers Cep164 and Odf2 (Fig. 5).

Figure 3d (0 MC). There still appears to be Cep63 foci. Please Explain.

Such tiny dim Cep63 puncta were also observed in the cells with one or two parental centrioles (Fig. 4d, revised manuscript). They might be Cep63 aggregates or non-specific background because they are distinct from the bright large centriolar foci of Cep63 and also do not co-stain with Cep152 and Centrin. Other centriolar markers also frequently exhibit similar tiny dim puncta. Therefore, when we need to identify centrioles or centriole-related structures, we always combined the co-staining patterns of at least two different markers. In the revised manuscript, we have included a sentence in the legends for Fig. 4d-e, Fig. 5a-b, and Fig. 6b-c and the quantification method (Materials and methods) to indicate the rationales.

References...Given how few papers there are published on deuterosomes the authors should be more inclusive to the field. Mori et al. stands out as a clear miss, while Mori et al. is referenced for a technical reason, their data actually strongly supports this paper and it seems like making a connection with that study would strengthen this paper. Also to mention "hyperactivated through overexpression of its key regulators" but not reference any of the numerous papers that have worked out the transcriptional mechanisms driving

this overexpression seems inappropriate. Other examples of important papers that have been left out are also present and a more thorough effort should be made.
Thanks for the comments. We have reorganized the introduction for clearer presentation and cited these important papers in the revised manuscript.

Referee #3:

Centrioles perform a dual function in eukaryotic cells, forming centrosomes in dividing cells and cilia in non-dividing cells. While many interphase cells possess a solitary cilium, nucleated by the older, mother centriole, certain terminally differentiated cells assemble up to several hundred motile cilia, whose coordinated beating helps generate fluid flow in the human respiratory tract, brain and oviduct. Assembly of these multiple cilia requires a fundamental change from the usual 'once and only once' mode of replication to generate the requisite number of centrioles. In vertebrates, centriole amplification in multiciliated cells involves both the assembly of multiple daughter centrioles on the same parent and assembly on non-centriolar generative structures called deuterosomes.
Here, Zhao and Chen propose that deuterosomes do not arise from association with the original parental centrioles as previously reported (Al Jord, Nature 2014), but 'de novo'. This certainly is a finding of sufficient interest for the readership of EMBO Reports. As detailed below, the data does not quite support such a sweeping conclusion. Nevertheless, I still support publication of this manuscript subject to a thorough revision of the text and the inclusion of some essential controls.

Major points

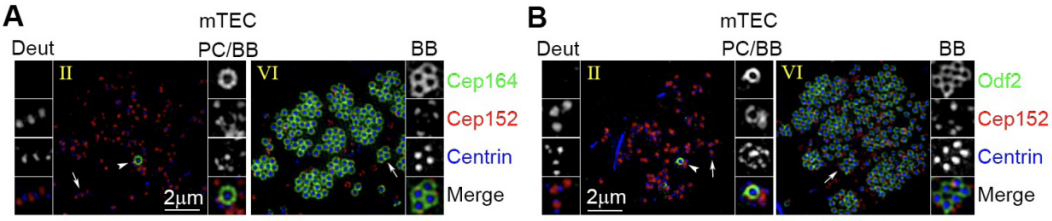
1. The authors interpret their results as a falsification of the main finding of Al Jord et al, that deuterosomes form in association with parental centrioles. This is not correct. If one discounts the argument made from the data in Figures 1 and 2 (which to me is ambiguous at best), the finding that deuterosomes can form in the absence of a parental centriole following Plk4 RNAi does not mean that they normally do so. The same argument could be made for canonical centriole duplication, which usually takes place in association with a parental centriole, but can also occur de novo following centriole removal (eg La Terra, JCB 2005, a literature well worth discussing). The evidence for centriole association of deuterosome formation in Al Jord et al, including live microscopy and EM, is quite strong and not invalidated by anything presented here.
We appreciate the insightful comments by this reviewer. In the revised manuscript, we have included the results of live cell imaging using GFP-Deup1 as deuterosome marker (Fig. 3; Movie EV1-2). We observed that deuterosomes emerge from a wide variety of locations in mEPCs, rather than from a certain position or small area implicated in the location of the daughter centriole. They initially appear as tiny dim foci, followed by a gradual increase in both size and GFP fluorescence intensity. Furthermore, multiple deuterosomes were seen to appear from different locations within minutes or an hour, which is in contrast to the report by Al Jord and colleagues that it takes at least two hours for a halo to form at and release from the daughter centriole. These results strongly suggest that deuterosomes are efficiently self-assembled even in the presence of parental centrioles. We have accordingly modified the main text.

2. Contrary to what the authors state (p4), Al Jord et al do not claim that procentrioles form only when deuterosomes are in the vicinity of a parental centriole or that deuterosomes are already mature (full size) upon release. As shown eg in Figure S6e of that paper, procentriole number increases upon release from the mother centriole and deuterosomes subsequently grow significantly larger. Showing much the same in Figures 1 and 2 and presenting this as evidence against mother centriole-'guided' deuterosome formation is therefore a straw man argument.
We thank the reviewer for the comments. We have deleted “after reaching the full size” in the revised manuscript. Regarding the procentriole formation, however, the Al Jord paper says that "all new centrioles derive from the pre-existing progenitor cell centrosome through multiple rounds of procentriole seeding (abstract)". In addition, the authors state that the centriole amplification "occurs in the vicinity of the centrosome (page 104, 1st paragraph, last

sentence)" and "is a recurrent process during which procentrioles are nucleated from the centrosomal daughter centriole to form halos that are released into the cytoplasm (page 105, 1st paragraph, last sentence)".
In the Fig. S6e, Al Jord and colleagues only examined "deuterosome volume distribution relative to their localization and the number of attached procentrioles (figure legend)" using electron micrographs. The results only indicate that "the procentriole-free deuterosomes were smaller than centrosome-bound deuterosomes loaded with procentrioles and, more generally, deuterosome size was correlated with the number of procentrioles (page 105, last sentence)". As the Al Jord paper reports that "only the daughter centrosomal centriole contributes to deuterosome formation (abstract)" and deuterosomes are released from the centriole as halos, we initially examined whether discrete deuterosomes indeed exist in the form of halos by performing experiments described in our Fig. 1-2. We found that this is not the case for the newly-formed discrete deuterosomes in both mTECs and mEPCs (Fig. 1-2). Therefore, we believe that the results in Fig. 1-2 establish the foundation of our study. In the revised manuscript, we have also revised the main text concerning the results of Fig. 1-2 for better clarity. We hope that the reviewer will find the presentation improved.

3. The strongest argument the authors present for centriole-independent deuterosome formation is their formation in cells lacking parental centrioles following Plk4 RNAi (Figures 3, 4). However, I'm not entirely convinced by the data presented for the absence of parental centrioles. Cep63/Cep164/Odf2 foci, though dim and somewhat too numerous, are visible in all 0MC cells (Figures 3d, 4a, b). Unexplained centrin streaks are also present in the same cells. Without EM, how can the authors be confident that centrioles are indeed not present? At the very least, the authors should present evidence (eg immunofluorescence microscopy with multiple centriolar markers) confirming that their antibodies indeed reliably and specifically detect centrioles.
The antibody to Cep63 was carefully characterized previously (Zhao et al., 2013). Immunoblotting In addition to the 3D-SIM images presented in the manuscript showing proper appendages-like staining of Cep164 and Odf2 (Fig. 5a,b), the antibodies to Cep164 and Odf2 also specifically labeled the basal bodies in stage-VI mTECs (please refer to the following Figure II). The antibodies also showed high specificity in western blotting (please refer to Figure III below).

Fig. II



Deut: deuterosome PC: parental centriole BB: basal body

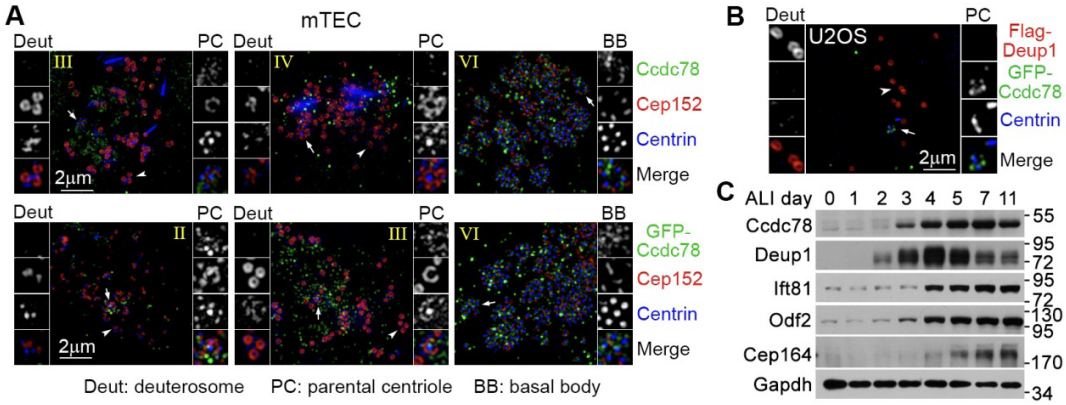
Although tiny dim puncta of Cep63/Cep164/Odf2 were often observed, the centriole-specific signals of these proteins can be clearly distinguished from the non-specific ones by the following rationales: their centriole-specific foci are large and bright, with shapes and centriolar locations identical to the well documented literature upon co-staining with Cep152 and GFP-Centrin1 (Fig. 4d and Fig. 5a,b). The dim tiny puncta apparently lack these features.
In our case, EM is unlikely a better way than 3D-SIM. Firstly, verifying cells with one or no parental centriole with EM requires continuous serial ultrathin sectioning and expert examinations of every section, which is unlikely practical when quantification results from large amount of cells are needed. Secondly, occasionally we could observe suspected procentriole formation on a small portion of deuterosomes in Plk4-RNAi cells at day 3. This is probably due to insufficient RNAi because *Plk4* is highly expressed in these cells (Fig. 3c) (Zhao et al., 2013). Such situations were more obvious in mEPCs treated with the Plk4 inhibitor centrinone (please refer to Fig. 6d). This adds another layer of complexity to EM because we have to distinguish such deuterosome-induced procentrioles from parental centrioles. In contrast, 3D-SIM with parental centriole markers can easily distinguish parental centrioles from procentrioles. We identify parental centrioles by combining the co-staining

patterns of at least two different markers. In the revised manuscript, we have included a sentence in the legends for Fig. 4d-e, Fig. 5a-b, and Fig. 6b-c and the quantification method (Materials and methods) to indicate this rationale.

Other points

4. The manuscript fails to provide an adequate overview of the existing literature on canonical centriole assembly (barely mentioned, but clearly relevant) and deuterosome-mediated centriole assembly. For the latter, at a minimum the following papers need to be cited and fully discussed: Klos-Dehring, Dev Cell 2013 (CCDC78 as a deuterosome protein), Mori, Nat Commun 2017 (E2f4 in de novo formation of deuterosomes), Vladar, JCB 2007 (establishment of mTECs as a model system for studying multiciliogenesis).
Moreover, a sentence like "We have previously found that the MC-mediated centriole amplification still uses the canonical ring-shaped platform around its basolateral wall, which contains the Cep63-Cep152-Plk4 complex and other components (Banterle & Gonczy, 2017, Brown, Marjanovic et al., 2013, Habedanck, Stierhof et al., 2005, Hatch, Kulukian et al., 2010, Nigg & Holland, 2018, Sir, Barr et al., 2011) but is hyperactivated through overexpression of its key regulators (Yan, Zhao et al., 2016, Zhao, Zhu et al., 2013), similar to what have been demonstrated in cycling cells by overexpressing Plk4, Cep152, SAS6, or STIL (Arquint, Sonnen et al., 2012, Dzhindzhev, Yu et al., 2010, Kleylein-Sohn, Westendorf et al., 2007, Strnad, Leidel et al., 2007, Vulprecht, David et al., 2012)." (p3, introduction) is not only overly long, but gives the impression that the authors are solely reponsible for our current understanding of centriole assembly in multiciliated cells. Similarly, "Fully assembled centrioles are eventually released from their "cradles" by APC/C-activated proteolysis and mature into basal bodies (Al Jord, Shihavuddin et al., 2017, Zhao et al., 2013)." (also p3) misleadingly suggests that the authors' 2013 paper contributed to the identification of a role for the APC/C in release of newly formed centrioles.
We appreciate these comments. In the revised manuscript, we have accordingly reorganized the introduction for better clarity and cited all the requested publications except for the one on Ccdc78.
Klos-Dehring and colleagues report that *Xenopus* Ccdc78 is a deuterosme protein in their Dev Cell paper. However, we can never detect any Ccdc78 on deuterosomes of mouse multiciliated cells (Please refer to the following Figure IIIA, arrowheads). Both endogenous Ccdc78 and exogenous GFP-Ccdc78 formed puncta irrelevant to deuterosomes in stages II-IV, but some puncta were observed at parental centriole (Figure IIIA, arrows). In stage VI, both Ccdc78 and GFP-Ccdc78 showed certain correlation with basal bodies (Figure IIIA). As exogenous Deup1 can induce deuterosome formation in cycling cells (Zhao et al.,2013), we also co-expressed Flag-Deup1 and GFP-Ccdc78 in U2OS cells and still found no Ccdc78 signals on the deuterosomes (Figure IIIB, arrowhead). GFP-Ccdc78 was again seen to co-stain strongly with a parental centriole (Figure IIIB, arrow). Furthermore, the expression pattern of Ccdc78 in differentiating mTECs is also distinct from centriole amplification regulators such as Deup1 but analogous to those of basal body appendage proteins such as Odf2 and Cep164 and ciliary proteins such as Ift81 (Figure IIIC). These results strongly argue that Ccdc78 is not a deuterosome protein, at least in mouse. We thus prefer not to cite the paper.

Fig. III



5. The authors give the impression that Al Jord et al were alone in suggesting deuterosomes arise in association with parental centrioles (p4, introduction) and that this would potentially be a unique feature of mouse ependymal cells. This is not so. For example, Kalnins and Porter (Z Zellforsch 1969) reported a mother centriole association of deuterosomes (here called cylindrical cores) in the chick tracheal epithelium.
We thank the reviewer for pointing this out. We have reorganized the introduction and cited the paper.

6. If deuterosome numbers per cell were assessed 'in cells containing deuterosomes' (legend to Figure 3g), does this not leave the possibility that there were Plk4-depleted cells that did not form any deuterosomes? This should be excluded by separately quantitating the number of cells without deuterosomes. Also, what criterion was used to assess deuterosome number - Deup1, Cep152?
Only a portion of the cultured ependymal progenitors can be induced to differentiate into multiciliated mEPCs (usually 30-60%) by serum starvation. We observed a reduced differentiation efficiency for Plk4-depleted progenitors (33.6%) as compared to the mock-depleted progenitors (52.1%) when the deuterosome-containing populations were quantified at day 3 (page 10, 2nd paragraph, revised manuscript). Such a reduction could be due to Plk4 depletion-induced self-renewal defects of the progenitors (Martin et al., 2014), because Deup1 levels were also reduced in the Plk4-depleted population (Fig. 4c, revised manuscript).
Deuterosomes were scored as ring-shaped structures decorated by both Deup1 and Cep152 or by Cep152 but excluding parental centrioles. We have included the criteria in the legends of Fig. 4g and Fig. 6e in the revised manuscript.

7. The authors only briefly mention that procentriole formation was abolished in Plk4-depleted cells (p9). Since this strengthens their argument that RNAi was effective in eliminating new centriole assembly (also of parental MCs), they may want to present proper quantiation of this. If not read carefully, the text gives the mistaken impression that Plk4 is not required for deuterosome-mediated centriole assembly (eg concluding sentence of abstract), which is not what the authors are seeking to claim.
Thanks for the suggestion. We have modified both the abstract (page 2) and the text (page 9, 2nd paragraph) in the revised manuscript to clearly indicate that Plk4 is essential for centriole biogenesis in both cycling cells and multiciliated cells. We have previously shown that the depletion of Plk4 with the same shRNA construct repressed deuterosome-mediated procentriole formation in mTECs (Zhao et al., 2013, Fig. 7f). In mEPCs, the repression was also efficient. GFP-Centrin1 foci were only occasionally observed to appear in the vicinity of deuterosomes to be procentriole suspects.
As stated in our reply to the point 3 of this reviewer, when centrinone was used to repress the Plk4 activity, the incidence of procentriole-like Centrin foci appeared to be increased at day 3 (Fig. 6d), despite its efficient depletion of parental centrioles in ependymal progenitors (day 0) (Fig. 6b,c). We attributed this to the failure for centrinone to inhibit the markedly elevated levels of Plk4 in these cells. As we focused on the contribution of parental centrioles on deuterosome formation, we did not elaborate the phenotype.

Minor comments

8. Labeling of insets in image panels is somewhat idiosyncratic and confusing. For example, in Figure 1c, which inset is showing Deup1, Cep152 and Centrin is only apparent from the multi-color merge. I do appreciate, though, that insets are presented in black and white for best contrast.
We have rearranged the insets in Fig. 1c,d to avoid confusing.

9. There seem to be a few instances of incorrect inset placement. Thus, the top set of insets in the middle lower panel of Fig. 4a shows Cep152 not Cep164 based on the merge. The same appears to be the case in the upper middle panel of Figure 4b (Odf2 vs Cep152).
We apologize for the mistakes. We have corrected these mistakes in the revised manuscript.

10. Quantifications of deuterosome diameter based on 3D-SIM images are presented with an unlikely number of significant figures (eg 211.1 +/- 59.9 nm, p5). Since measured particle size depends on image thresholding, the methods section needs to be more clear how this was determined.
We used the default setting of the 'count /size' function of Image-Pro Plus 6.0 software. We have stated this in the Method in the revised manuscript.

11. Centrin is an unfortunate, if frequently used, marker for centrioles, since it also localizes to centriolar satellites (Dammermann, JCB 2002; Hori, MBOC 2015). This likely explains the 'aggregates' observed by the authors in mEPCs (p6).
Thanks for the suggestion and have cited the papers in our revised manuscript. Centrin is a widely used as a pan-centriole marker. But indeed its non-centriole fluorescent signals are sometimes a nuisance.

12. 'punctual' (p6) should read 'punctate'.
We have corrected the typo. Thanks.

13. The liberal use of acronyms and unusual abbreviations is making this manuscript unnecessarily difficult to read. It's bad enough to have mother centrioles referred to throughout as MCs. But who remembers that 'SS d2' means 2 days post serum starvation or that Plk4i/Ctrli-expressing mEPCs are Plk4/Control RNAi-depleted cells? MCD and DD (Figure 1a, presumably mother centriole and deuterosome-dependent centriole assembly pathways) are nowhere defined in the paper.
We thank the reviewer for the comments. In the revised manuscript, we have limited the use of abbreviations for better readability.

2nd Editorial Decision 9 January 2019

Thank you for the submission of your revised manuscript to EMBO reports.

Referee 1 was unfortunately not available anymore but we have meanwhile received the reports from referee 2 and 3. We note that referee 2 remains rather skeptical regarding the general interest of your findings, but given the support from referee 3 (and referee 1 in the first round of reviews), we have decided to invite you to further revise your manuscript for publication in EMBO reports. Upon further discussion, referee 3 agrees with referee 2 that centrin is not the ideal centriolar marker but nevertheless widely used in the field. This referee also acknowledges that you use other centriolar markers as well to support your conclusions. Please address all other remaining concerns from referee 1 and 3 in the text. All relevant literature should be cited in the most appropriate manner. Please note that also articles published on bioRxiv can be cited and discussed and I suggest including and discussing the two recent articles on centriole amplification.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your manuscript:

REFEREE REPORTS

****

Referee #2:

The current version of the manuscript by Zhao has some improvements including the addition of centrinone treatment for the loss of parental centrioles. The data in this paper is certainly well done. My concern all along was whether the advance was worthy of publication in EMBO. This is a descriptive paper that does not provide any mechanistic detail into how, when or where deuterosomes arise. It simply describes the localization and shows that in contrast to another publication (al Jord) that localization does not require parental centrioles. This is an important clarification, to be sure, but one that I think is appropriate for a more specialized journal.

I appreciate the authors attempt at describing the localization of centrin. I am not arguing against it being in cilia, it is just that it is surprising how strong it is. Others have certainly seen centrin in cilia but it is always noticeably stronger in basal bodies/centrioles, which is not the case here. I stand by my concern that centrin is used as a marker for cilia, centrioles and nothing in different places in this paper. While the authors are most likely right in their interpretation we are left trusting their judgment rather than having clear markers.

While I found the more thorough referencing of the literature to be markedly improved, I also found the author's refusal to references Klos et al. as requested by reviewer 3 to be unacceptable. The goal of the field is broader than just mouse. If there is a discrepancy in the CCDC78 localization between systems that might be quite interesting, but I appreciate that this is not the goal of this paper. At the very least that paper was the first to implicate Cep152 in the deuterosome mediated generation of centrioles and should be referenced. Given the small number of papers relevant in this field this paper should be referenced for its contribution even if the authors are not pursuing CCDC78 specifically.

While the authors tout the addition of movies showing Deup1 foci formation as definitive proof of de novo synthesis I think this is overstated. Without having a marker for the parental centrioles it is a bit hard to evaluate the relative location of deuterosome nucleation. I agree that it is unlikely that the centrosome is jumping all over the place, however, without actually visualizing it I am not sure one can say that it is definitively NOT. Additionally, it is possible that deuterosomes form prior to recruitment of Deup1 or at least prior to the accumulation of enough Deup1 for visualization.

Referee #3:

In preparing this revision, the authors went above and beyond to satisfy the reviewers' concerns, with major changes to the text and the inclusion of additional experiments, including live imaging of deuterosome assembly and PLK4 inhibition using centrinone. These experiments significantly strengthen the authors' argument of centriole-independent deuterosome assembly. I therefore consider this manuscript suitable for publication in EMBO Reports subject to minor changes to the text and figures.

Essential
1. The manuscript still does not present all of the relevant literature. For a paper demonstrating the de novo assembly of deuterosomes when parent centrioles are removed to not discuss the de novo assembly of centrioles as occurs eg following centriole ablation (La Terra, JCB 2005) is a glaring omission. The authors are free to point out any differences (based on their work deuterosomes form de novo also in the presence of parental centrioles). Still, it is an important comparison to make. Similarly, the work of the Mitchell lab on CCDC78 as a deuterosome protein in Xenopus (Klos-Dehring, Dev Cell 2013) deserves to be cited. Again, the authors are free to point out that in their hands in mice CCDC78 does not appear to be a deuterosome protein, either as data not shown or presenting their reviewer figure as a supplemental figure. This is important information, and would

be good to have out there in the literature.

2. The manuscript continues to present measurements with implausible numbers of significant figures (0.1nm) which are impossible to obtain from 3D-SIM data. Further, referring to 'the default setting of the 'count /size' function of Image-Pro Plus 6.0 software' does not address how image thresholding/filtering was done, which has profound effects on the values obtained (see the Image-Pro Plus user manual).

3. Black & white insets continue to be presented without labels, leaving the reader to guess which is which from the color merge or the order of labels on the panel heading. I understand that the authors are trying to avoid cluttering their images with text, but even a roman numeral (I,II,III) would be helpful in interpreting panels such as in Fig 2b.

4. p9 "As expected, procentriole formation was abolished in these cells (Fig. 3d,f)." p12 "In addition, some procentrioles appeared to form on deuterosomes in a portion of centrinone-treated cells at day 3 (Fig. 6d), possibly due to the failure of the centrinone to inhibit the markedly elevated levels of Plk4 in these cells (Fig. 3c) [36]"
I believe the authors would benefit from more clearly stating (and ideally presenting quantitative data to support this) the extent to which PLK4 RNAi/centrinone treatment prevented centriole assembly subsequent to deuterosome formation, both from deuterosomes and any remaining parental centrioles, as this strengthens their case of adequate PLK4 depletion/inhibition. Referring to a previous study of theirs does not adequately make that point. This is of some interest as other groups are now reporting that PLK4 may be dispensable for centriole amplification in multiciliated cells.

Recommended
5. p8/9 "When imaged in the presence of nocodazole, 15 cells that were undergoing de novo deuterosome biogenesis were observed (Fig. 3e and Movie EV2), whereas another 16 cells displayed increasing number of deuterosomes over time."
What does this mean? 16 cells did not show de novo biogenesis, or were not imaged in the same way as the other 15?

6. The label PLK4i is missing from panel Fig 5b.

2nd Revision - authors' response 28 January 2019

Responses to reviewers' concerns

Referee #2:

The current version of the manuscript by Zhao has some improvements including the addition of centrinone treatment for the loss of parental centrioles. The data in this paper is certainly well done. My concern all along was whether the advance was worthy of publication in EMBO. This is a descriptive paper that does not provide any mechanistic detail into how, when or where deuterosomes arise. It simply describes the localization and shows that in contrast to another publication (al Jord) that localization does not require parental centrioles. This is an important clarification, to be sure, but one that I think is appropriate for a more specialized journal.
We thank the reviewer for appreciating the importance and quality of our study and considering our manuscript publishable but regret that the reviewer did not fully realize the importance of our study. The mechanistic details on how, when, and where deuterosomes arise are answered in our previous publication using mTECs as a model system (Zhao et al., 2013). Our current studies do not "simply" describe the localization of deuterosomes. Rather, we have clarified important issues raised by other researchers on the origins of deuterosomes and their associated procentrioles.

I appreciate the authors attempt at describing the localization of centrin. I am not arguing against it being in cilia, it is just that it is surprising how strong it is. Others have certainly seen centrin in cilia but it is always noticeably stronger in basal bodies/centrioles, which is not the case here. I stand by my concern that centrin is used as a marker for cilia, centrioles and nothing in different places in this

paper. While the authors are most likely right in their interpretation we are left trusting their judgment rather than having clear markers.
We in fact use acetylated tubulin as the standard ciliary marker in our study (Figures 5C and EV3). Centrin is always used as a centriolar marker. Furthermore, we use at least two different markers when a clear discrimination of different centrioles is necessary. Centrin usually distributed more prominently in the long primary cilia of differentiating mEPCs than in the short cilia of the precursor cells (Figure 5). Although these are interesting observations, we hope that the reviewer would agree that understanding why Centrin displays such localizations is beyond the scope of our current study.

While I found the more thorough referencing of the literature to be markedly improved, I also found the author's refusal to references Klos et al. as requested by reviewer 3 to be unacceptable. The goal of the field is broader than just mouse. If there is a discrepancy in the CCDC78 localization between systems that might be quite interesting, but I appreciate that this is not the goal of this paper. At the very least that paper was the first to implicate Cep152 in the deuterosome mediated generation of centrioles and should be referenced. Given the small number of papers relevant in this field this paper should be referenced for its contribution even if the authors are not pursuing CCDC78 specifically.
We respect the reviewer's comments and have cited the paper in this revised manuscript. We have also included our results shown in the previous reviewer figure as Figure EV1, as suggested by our reviewer #3 and modified the main text accordingly.
Whether the paper is "the first to implicate Cep152 in the deuterosome mediated generation of centrioles" depends on what is considered as "the first". Our paper (Zhao et al., 2013) was submitted to Nat Cell Biol on April 15, 2013, whereas the Ccdc78 paper was submitted to Dev Cell on July 26, 2013, when our manuscript was in the middle of revision. Nevertheless, this is not why we chose not to cite the paper in our previous manuscripts. As Deup1 functions in centriole amplification in Xenopus (Zhao et al., 2013), it is unlikely that Ccdc78 only functions in Xenopus deuterosomes but not in mammalian ones. Everyone knows that arguing against colleagues' conclusion is a difficult job. We just did not intend to do it twice in one manuscript.

While the authors tout the addition of movies showing Deup1 foci formation as definitive proof of de novo synthesis I think this is overstated. Without having a marker for the parental centrioles it is a bit hard to evaluate the relative location of deuterosome nucleation. I agree that it is unlikely that the centrosome is jumping all over the place, however, without actually visualizing it I am not sure one can say that it is definitively NOT. Additionally, it is possible that deuterosomes form prior to recruitment of Deup1 or at least prior to the accumulation of enough Deup1 for visualization.
We agree that our live imaging is not perfect due to limitations in techniques as well as time. We thus only carefully state our observations in Figure 3 with sentences such as "nascent deuterosomes emerge from a wide variety of locations in mEPCs " (page 10, line 5) and such results "strongly suggest that deuterosomes self-assemble efficiently" (page 11, line 13). To further strengthen this, we have included an additional movie (Movie EV3) in this revised manuscript, in response to the editor's request to present results previously marked as "data not shown". As the reviewer points out, the centrosome is unlikely jumping all over the place in the cells. The nice time-lapse images by Al Jord and colleagues indeed show that parental centrioles are closely located in the nuclear area and slowly oscillate in early phase of the centriole amplification (Al Jord et al., 2014). Despite this, we have never tried to use our live imaging results as "definitive proof of de novo synthesis". Therefore, we do not think that we have overstated our results.
Our definitive proof of de novo deuterosome biogenesis comes from the results in Figures 4, 5, and EV4, in which we show that efficient deuterosome formation persists after the depletion of both parental centrioles. Furthermore, our results are supported by two recent bioRxiv preprints, one by Nanjundappa and colleagues using centrinone-treated mTECs and the other by Mercey and colleagues, from the same group that has reported the daughter centriole-dependent mechanism, using centrinone-treated mEPCs. As suggested by the editor, we have cited these two preprints in our revised manuscript (page 15, line 10).
Finally, we have previously demonstrated that Deup1 is essential to deuterosome formation (Zhao et al., 2013), excluding the possibility that "deuterosomes form prior to recruitment of Deup1 or at least prior to the accumulation of enough Deup1 for visualization" at the daughter centriole. In addition, we hope that the reviewer has noticed that, in our manuscript, we have never excluded the possibility that parental centrioles can also serve as deuterosome nucleation sites in intact cells.

Referee #3:

In preparing this revision, the authors went above and beyond to satisfy the reviewers' concerns, with major changes to the text and the inclusion of additional experiments, including live imaging of deuterosome assembly and PLK4 inhibition using centrinone. These experiments significantly strengthen the authors' argument of centriole-independent deuterosome assembly. I therefore consider this manuscript suitable for publication in EMBO Reports subject to minor changes to the text and figures.
We thank the reviewer for appreciating our efforts in the revision.

Essential

1. The manuscript still does not present all of the relevant literature. For a paper demonstrating the de novo assembly of deuterosomes when parent centrioles are removed to not discuss the de novo assembly of centrioles as occurs eg following centriole ablation (La Terra, JCB 2005) is a glaring omission. The authors are free to point out any differences (based on their work deuterosomes form de novo also in the presence of parental centrioles). Still, it is an important comparison to make. Similarly, the work of the Mitchell lab on CCDC78 as a deuterosome protein in Xenopus (Klos-Dehring, Dev Cell 2013) deserves to be cited. Again, the authors are free to point out that in their hands in mice CCDC78 does not appear to be a deuterosome protein, either as data not shown or presenting their reviewer figure as a supplemental figure. This is important information, and would be good to have out there in the literature.
In the revised manuscript, we have included the topic of de novo centriole assembly and cited the requested and other relevant papers (page 15, line 12). We have also presented our data on Ccdc78 as Figure EV1 and modified the text accordingly.

2. The manuscript continues to present measurements with implausible numbers of significant figures (0.1nm) which are impossible to obtain from 3D-SIM data. Further, referring to 'the default setting of the 'count /size' function of Image-Pro Plus 6.0 software' does not address how image thresholding/filtering was done, which has profound effects on the values obtained (see the Image-Pro Plus user manual).
As the optical resolution of 3D-SIM is approximately 120 nm, we have used rounding to two significant figures for the mean and s.d. values in the revised manuscript. As to thresholding/filtering, we used the "automatic bright objects" mode of the “count/size” function of Image-Pro Plus 6.0. As the Deup1 epifluorescence is very bright, the automatic mode recognizes deuterosome boundaries well. We have included the information in the revised manuscript.

3. Black & white insets continue to be presented without labels, leaving the reader to guess which is which from the color merge or the order of labels on the panel heading. I understand that the authors are trying to avoid cluttering their images with text, but even a roman numeral (I,II,III) would be helpful in interpreting panels such as in Fig 2b.
In the revised manuscript, we have used labels p1, p2, and/or dt to indicate parental centrioles and representative deuterosomes in Figures 1C/D, 2A/B/E, 5A-C, EV1A/B/C, and EV4D to aid understanding. We have also arranged all the insets in Figure 2B in a left-to-right manner and indicated the order of the insets in the figure legend to avoid confusion. We have similarly indicated the order of the insets in Figure 2E.

4. p9 "As expected, procentriole formation was abolished in these cells (Fig. 3d,f)." p12 "In addition, some procentrioles appeared to form on deuterosomes in a portion of centrinone-treated cells at day 3 (Fig. 6d), possibly due to the failure of the centrinone to inhibit the markedly elevated levels of Plk4 in these cells (Fig. 3c) [36]"
I believe the authors would benefit from more clearly stating (and ideally presenting quantitative data to support this) the extent to which PLK4 RNAi/centrinone treatment prevented centriole assembly subsequent to deuterosome formation, both from deuterosomes and any remaining parental centrioles, as this strengthens their case of adequate PLK4 depletion/inhibition. Referring to a previous study of theirs does not adequately make that point. This is of some interest as other groups are now reporting that PLK4 may be dispensable for centriole amplification in multiciliated cells.

We have modified the text as requested. In Plk4 RNAi mEPCs, procentrioles were not observed on deuterosomes as far as we can judge (n=100 cells), consistent with our previous results in mTECs (Zhao et al., 2013). In centrinone-treated mEPCs (n=97), however, 97% had procentriole-associated deuterosomes.

Recommended

5. p8/9 "When imaged in the presence of nocodazole, 15 cells that were undergoing de novo deuterosome biogenesis were observed (Fig. 3e and Movie EV2), whereas another 16 cells displayed increasing number of deuterosomes over time."
What does this mean? 16 cells did not show de novo biogenesis, or were not imaged in the same way as the other 15?
We captured 15 cells that initiated their deuterosome biogenesis during the imaging and 16 cells that already contained deuterosomes from the beginning and showed increased numbers of their deuterosomes over time. We have clarified this in the revised manuscript (page 11, line 1-4). Furthermore, we have included an additional movie (Movie EV3) to represent the 16 cells, in response to the editor's request.

6. The label PLK4i is missing from panel Fig 5b.

Sorry for this. We have fixed the problem.

3rd Editorial Decision 6 February 2019

Thank you for submitting your revised manuscript to EMBO Reports. I have now looked at everything and all looks fine. Therefore I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on the very nice work!

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Xueliang Zhu
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2018-46735V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No sample-size calculation was performed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No animal study in this paper.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No method of randomization was used.
For animal studies, include a statement about randomization even if no randomization was used.	No animal study in this paper.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Cells were randomly selected only based on the treatment.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No animal study in this paper.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The unpaired t-test was performed to access differences with no assumption of our data. If failed, the Mann-Whitney test was then applied to detect differences.
Is there an estimate of variation within each group of data?	Yes. The estimate of variation was shown by using standard deviation (SD).
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The antibodies have been accurately annotated.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T cells were from ATCC and were routinely tested for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	4-week old and P0 C57BL/6J mice were used for primary cell culture. The source of mice were provided in the "materials and Methods".
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The statement was included in the "Materials and Methods".
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.