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The dual role of the centrosome in organizing the microtubule network in interphase

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Transaction Report: This manuscript was transferred to *EMBO reports* following review at *The EMBO Journal*

1st Editorial Decision 14 February 2018

Thank you for the transfer of your research manuscript to EMBO reports. As I had indicated earlier, I would like to give you the opportunity to submit a revised manuscript to EMBO reports, which addresses the key technical points raised by the referees who evaluated your study for The EMBO Journal. Moreover, the revision should include a somewhat more detailed characterization of cytoplasmic microtubule nucleation as outlined by referee 1.

In particular, the revision should address the following points experimentally:
- Validate the completeness of AKAP450 and PCNT KO as outlined by referee 1.

- Address the effects on microtubule mass/tubulin expression (referee 2 and 3). You already indicated that you can quantify the concentration of alpha-tubulin in centrosome-less cells compared to non-treated cells.

- Add a more detailed description of cytoplasmic microtubule nucleation detected in the absence of centrosome, PCNT and AKAP450 as outlined by referee 1. It will not be necessary to determine the underlying mechanism in more detail, though.

- Provide some data on the role of Cep192 in MT nucleation at the centrosome such as gamma-tubulin association with the centrosome and MT nucleation capacity in the absence of Cep192. This will provide an independent confirmation of your hypothesis as outlined by referee 2.

Please address these and all other 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 and I will aim to secure the same referees that evaluated your study for The EMBO Journal. 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.

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- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
(In order to avoid delays later in the publication process, please check our figure guidelines before preparing the figures for your manuscript:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf)
- a separate PDF file of any Supplementary information (in its final format)
- all corresponding authors are required to provide an ORCID ID for their name. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (http://embor.embopress.org/authorguide).

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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.

1st Revision - authors' response 15 May 2018

Referee #1:

The authors have extensively analysed the effect of centrosome gain/loss and the depletion of three proteins, pericentrin (PCNT), CDK5RAP2, and AKAP450, which are known to bind to the gamma-tubulin complex, the major microtubule nucleator. The experimental strategy was straightforward: the authors generated KO lines for these proteins, both individually and in combinations, and observed microtubule formation and organisation at the centrosome and Golgi, two major microtubule nucleation centres during interphase, using immunofluorescence microscopy. Elimination or amplification of centrosomes was achieved by treatment or washout of the Plk4 inhibitor centrinone, respectively.

The work reported in this study has a certain degree of overlap with that in a previous report by the Akhmanova group, including triple inhibition of AKAP450, CDK5RAP2 and the centrosome (Wu et al. Dev Cell. 2016). Not surprisingly, some results are consistent between these two studies. However, while Wu et al. focused on the analysis of microtubule nucleation as well as organisation at the Golgi, the current study has analysed the centrosomal nucleation in detail. I can also see several new elements in the current study, such as artificial overexpression of centrosomes or characterisation of the PCNT KO line.

I was most excited at the discovery of two modes of cytoplasmic microtubule nucleation in the conventional tissue culture cell line. PCNT-dependent cytoplasmic MTOCs, which were detected in the absence of centrosome- and Golgi-dependent nucleation, resemble MTOCs observed in mouse oocytes and early embryos (e.g. Courtois et al. J Cell Biol. 2012). More strikingly, cytoplasmic microtubules were generated even without PCNT in this condition, which is, to my knowledge, a novel finding in animal cells; it may be similar to what has been observed in plant cells, which do not possess centrosomes or Golgi-dependent nucleation (Nakaoka et al. Plant Cell. 2015). The current study could be better presented by highlighting these totally new elements. My suggestions are as follows:

1. Cytoplasmic nucleation
The authors should characterise the PCNT-independent cytoplasmic microtubule nucleation in more detail; in my opinion, this is one of the most striking findings in this study. At the minimum, they should stain gamma-tubulin together with microtubules and clarify the mode of nucleation; do microtubules exhibit mini-asters (like cMTOCs) or branched configuration (like dominantly observed in cortical microtubule arrays in plants [e.g. Liu et al. Curr Biol. 2014]), or is a single microtubule spontaneously nucleated just like in the plant endoplasm (Nakaoka et al. Plant Cell. 2015)? The detailed information would enable a more thorough and interesting discussion on the microtubule nucleation from the evolutionary perspective, as the current study might have settled the core mechanism of microtubule nucleation in eukaryotes.
We have now included a more detailed description of the PCNT-independent cytoplasmic MT nucleation. Our results indicate that MTs grow as single units, in contrast with MT growing as mini-asters from PCNT-containing cMTOCs. We were unable to visualize g-tubulin at the minus end of cytoplasmic MTs in fixed cells. As far as we know, in non-polarized mammalian cells g-tubulin labelling is hard to observe out of the centrosome. Alternatively, we have treated centrosome-free *akap* KO cells depleted or not of PCNT with the g-tubulin inhibitor gatastatin that has been reported to partially inhibit and delay MT nucleation (Chien et al., Nat Comm 2015). Our results demonstrate that g-tubulin is involved in both PCNT-dependent and independent cytoplasmic MT nucleation.
In summary, we show in the new version that PCNT-independent cytoplasmic microtubule nucleation is a g-tubulin dependent process able to generate cell-wide MT networks from individual MTs.

2. KO line (Fig. 1 and Fig. S1)
My major technical concern is the completeness of knockout. The KO lines were selected by introducing a frame-shift at an early exon, not by replacing the entire open reading frame with a selection marker. This treatment leaves the possibility that a shorter isoform is expressed and functions as the gamma-tubulin regulator. The authors suggested that this might indeed be the case for AKAP450. They should also test this possibility for other two factors. Western blotting is not the best analysis technique to exclude the possibility, since antibodies generally recognise non-specific bands also. I would suggest immunostaining of the target protein with an antibody recognising the middle or C-terminal region of the protein. The authors have shown this in Fig. S1D, but images with higher contrast (longer exposure time) and co-stained with markers of centrosome or Golgi are required. When I adjusted the brightness/contrast of the provided image, I could see some punctate signals of CDK5RAP2, which might represent the residual CDK5RAP2. Since some of the authors' conclusions are based on the "negative" result (i.e. no apparent defect in the KO line), information regarding the completeness of the KO is critical.
We have now tested our AKAP450 and PCNT knock-out cell lines with two additional antibodies raised against the C-terminal part of the respective protein as proposed by the reviewer. The new goat anti-AKAP450 antibody (Ct-AK; MyBioSource, MBS420313) recognizes aas 3887-3898 of the protein. The new rabbit anti-PCNT antibody (Ct-PCNT; Biomatik, USA) was raised against aminoacids 2987-3246. In wild-type cells, Ct-AK antibody labelled both the GA and the centrosome while Ct-PCNT labelled the centrosome as expected. However, no signal was detected in the respective KO cell line (new Fig. EV1) confirming the absence of putative C-terminal containing isoforms. We have also modified Fig. S1D (new Fig. EV4) according to referee's suggestion.
We have used in this study four anti-AKAP450, four anti-PCNT and three anti-CDK5RAP2 antibodies that recognized epitopes distributed all over the respective protein sequence (Fig. 1A). We have tested two clones of each KO cell line with all of them by IF and WB (when possible). We have not detected any signal either by WB or IF. The only exception was the polyclonal anti-AKAP450 antibody from Aviva that although raised against the N-terminal part of AKAP450, also recognized the third quarter of the protein (marked as Av and Av* epitopes in Fig. 1A; see also Fig. EV3D). This antibody displayed residual centrosomal labelling in *akap* KO cells. Since the new C-terminal specific antibody we used (Ct-AK) did not reveal any signal at the centrosome in any of the two KO clones, in our opinion, this residual centrosomal signal is due to cross-reaction of the polyclonal Av antibody with a centrosomal epitope rather than to the expression of a minor centrosomal isoform. In addition, anti-centrosomes antibodies are frequently present in rabbit pre-immune sera that could explain the residual signal.
We have also performed qPCR experiments to quantify mRNA levels of each mutated gene. We have used three (for either AKAP450 or PCNT) or two (for CDK5RAP2) pairs of primers distributed along the RNA sequence as represented in Fig. EV3. mRNA levels decreased by more than 90% in all cases in agreement with CRISPr/Cas9 mutated transcripts being degraded by 5'-3' non-sense mediated mRNA decay (Popp and Maquat, Cell, 2016). Thus, as far as we can tell, this analysis demonstrates the reliability of the KO cell lines we generated.

3. Immunoprecipitation (Fig. 3A). To exclude the possibility of cross-reaction of the antibody (e.g. anti-AKAP450) with the co-precipitated protein (e.g. CDK5RAP2), the KO line should be used as the control (e.g. AKAP450 KO line for anti-AKAP450 IP).
In our experiments, slices of western blots containing proteins of appropriate molecular weight were incubated with each antibody, i.e., slices containing proteins with MW higher than 300 kDa were incubated with the anti-AKAP450 antibody, those containing proteins of MW ranging from 150 to 300 kDa were incubated with anti-CDK5RAP2 and the rest of the membrane was incubated with an anti-g-tubulin antibody. For detection of PCNT and AKAP450 (Fig. 3B), duplicated western blots were used. So, we can exclude the possibility of cross-reaction.

4. Line 276: "numerous spots concentrated around the GA" cannot be seen well in this figure panel.
Improved

5. Line 81: In the abstract and discussion, the "default" claim is made based on the PCNT-deletion experiment, whereas it was concluded here based on PCNT-containing aster formation. This should be corrected. Corrected

Referee #2:

Microtubule organizing centers (MTOCs) nucleate and organize microtubules at specific sites in the cell to generate spatial patterns required for different cell functions. In this manuscript, Gavilan et al. explore the relationship between different MTOCs, in particular the centrosome and the Golgi in RPE1 cells. The authors look at the MTOC localization and requirement for three g-tubulin recruiting factors, PCNT, CDK5RAP2, and AKAP450, all of which localize to both the centrosome and the Golgi. Using CRISPR KO strains, the authors find that removal of AKAP450 or CDK5RAP2 impairs MT nucleation at the Golgi and has a small, but statistically significant effect on MT nucleation at the centrosome. Removal of PCNT decreases MT nucleation at the centrosome, but leads to increased MT nucleation at the Golgi, increased AKAP450 and CDK5RAP2 at the Golgi and increased AKAP450 at the centrosome. The authors find that centrinone, a PLK4 inhibitor that leads to the removal of centrosomes over a few cell cycles, treatment leads to increased AKAP450 dependent Golgi MT nucleation, increased AKAP450, CDK5RAP2, and PCNT at the Golgi, and increased total a-tubulin concentration in cells. Furthermore, the removal of the centrosome and AKAP450 to perturb both centrosomal and Golgi MT nucleation results in the appearance of PCNT-dependent cytoplasmic MTOCs (cMTOCs). The authors propose a hierarchy of MTOC function: centrosome > Golgi > cMTOCs.
The results presented here are intriguing, but will require additional analyses to be substantiated and to lead to specific mechanisms underlying this proposed hierarchical relationship.

Major Comments:
-One of the main conclusions presented here rests on the use of centrinone. However, little is known about the effect of centrinone on the rest of the cell. Of particular importance is to understand if centrinone has any impact on 1) the Golgi itself or 2) to overall tubulin concentrations in the cell. Both of these points are particular germane to understand whether the observed phenotypes are due to action of the centrosome per se rather than indirect effects of centrinone on the cell.
The effect of centrinone on the GA was originally reported in Wong et al., Science 2015 and further studied by Wu et al., Dev. Cell, 2016. Strikingly, they found that a single perinuclear Golgi ribbon can be assembled in the absence of centrosomes. Wu et al., 2016 also investigated the Golgi structure of centrinone-treated cells by EM and found that neither the size nor the shape of individual Golgi stacks were modified by the absence of the centrosome. They measured length and width of individual Golgi stacks and did not detect any differences.
To exclude an effect of centrinone in overall tubulin concentrations, we have measured a- and b-tubulin levels of control and centrinone-treated cells by WB and normalized the values with respect to either Hsp70 content or total protein level (Fig. EV7). No substantial differences were detected in any case as shown in Fig. EV7.

The authors find that acute treatment with centrinone (3-12 hours) does not impact nucleation at the Golgi. Similarly, Wong et al., 2015 (referee is probably referring to data from Wu et al 2016) and these studies find that centrinone treatment for longer periods does not impact regrowth and in fact seem to increase MT nucleation at the Golgi. Although the authors show that the presence and position of the Golgi does not seem to be impacted by centrinone, it is important to understand whether the number of Golgi ribbons or fragments is impacted. Increased Golgi numbers would provide increased sites for MT nucleation.
As reported by Wu et al. 2016 and confirmed by us in this work, centrosome-free cells are larger than control cells and, in parallel, the GA is also bigger. As mentioned above, Wu et al., 2016 showed that neither the size nor the shape of individual Golgi stacks was modified by the absence of the centrosome but they did not quantify the number of Golgi stacks. Anyway, we have carefully quantified both the number of Golgi elements able to nucleate MTs in centrinone-treated cells compared to wild-type cells and the number of MTs growing from each Golgi element under both conditions. Our results demonstrate that both parameters consistently increased in the absence of centrosomes. In Fig. 4A, it is easily appreciable that Golgi stacks of similar size nucleated more MTs when the centrosome is absent.
It is also worth mentioning that centrinone-removal induced high number of centrosomes inhibited MT nucleation at the GA (see Fig. 4). These experiments were carried out 48 h after centrinone release, when the effect of the drug on the GA, if any, should have disappeared.
Related to this point, it is not clear whether quantitation of Golgi localization of g-TuRC localizing factors or of MTs is normalized against a Golgi marker. Increased Golgi numbers would also then effect these measurements.

Quantifications shown in Fig. 5B could not be done using a standard co-localization software. AKAP450, CDK5Rap2 and PCNT accumulated as dots juxtaposed to Golgi elements but the colocalization area between each one and a Golgi marker was very low. This can be clearly appreciated in magnifications in Fig. 5B. Therefore, we identified Golgi elements and quantified the intensity of either AKAP450, CDK5Rap2 or PCNT IF signals associated with them. Individual Golgi elements from at least 14 cells were delineated (>500 elements/experiment) and the intensity of each protein spot associated with each Golgi element was measured.

2) The authors conclude that microtubule polymer mass is increased following centrinone treatment, but conclude this based on quantifying "total a-tubulin fluorescence," rather than numbers of individual polymers. Based on the data, it is plausible to conclude that centrinone treatment increase tubulin expression, which would itself lead to an increase in microtubule nucleation at different MTOCs. It is important to understand whether the loss of the centrosome per se or whether a total increase in tubulin levels is responsible to the general increase in microtubule nucleation from the Golgi.
As mentioned above "To exclude an effect of centrinone in overall tubulin concentrations, we have now measured a- and b-tubulin levels of control and centrinone-treated cells by WB and normalized the values with respect to either Hsp70 content or total protein level (Fig. EV7). No substantial differences were detected in any case as shown in Fig. EV7. "

- The authors suggest a model where the centrosome sequesters components necessary for MT nucleation at the Golgi and cMTOCs, i.e. PCNT and AKAP450.
One prediction of this model is that these components would be limiting. However, loss of PCNT leads to increased accumulation of AKAP450 at the centrosome and the Golgi (minimal, only 8% of increase) and a concomitant increase in MT nucleation at the Golgi. Thus, increased centrosomal accumulation of AKAP450 does not inhibit Golgi MTOC function, but rather increases it.
We have now further clarified this point that admittedly was confusing in the precedent version. We do not think that AKAP450 is a limiting factor by itself since it is a very abundant protein. Furthermore, the GA is much larger than the centrosome and the amount of Golgi-bound AKAP450 is much higher than that of the centrosome. However, AKAP450 (and PCNT) acts as a signaling platform that binds kinases, phosphatases, phosphodiesterases, etc. For example, a PKA-PDE4D3-AKAP450 complex was reported to generate spatial compartmentalization of cyclic cAMP signaling at the centrosome (Terrin et al., JCB 2012). In our vision, regulatory component(s) of these complexes could be acting as limiting factor(s). More work is necessary to understand this apparently complex regulation.

Another prediction of this model (and an independent measure aside from centrinone treatment) would be that overexpression of the centrosomal PACT domain could compete off full length PCNT and AKAP450 from the centrosome and similarly lead to increased MT nucleation at the Golgi.
In our hands, overexpression of the PACT domain does not displace endogenous AKAP450 or PCNT from the centrosome suggesting that these proteins establish other interactions in the PCM. In fact, tagging with the PACT domain is a widely employed method to target any protein to the centrosome, but the receptors are not characterized. It is generally assumed that this does not perturb centrosome functioning nor affect AKAP450 and PCNT distribution.
- A prediction of these data is that the loss of nucleating capacity at the centrosome would also lead to increased Golgi MT nucleation. Thus, analysis of MTs after depletion of a PCM component such as CEP192 would be an independent confirmation of the model. Similarly, does NEDD1 removal lead to the same Golgi phenotype, or is this really a competition for PCNT and AKP450?
We have included siRNA CEP192 experiments in the new version of the manuscript. We have quantified EB1 comets in control cells (Fig. 2E) and EB1 signal intensity in MT regrowth assays (Fig. 2F) in cells depleted of CEP192. We have found that MT nucleation rate from the centrosome was reduced by 28.8% and 35.5%, respectively, in the absence of CEP192. We also tested depletion of CEP192 in double PCNT-AKAP450 KO cells, that also lack CDK5Rap2. Under these conditions, MT nucleation decreased by 42.25% (measuring EB1 comets) or 53.4% (quantifying EB1 intensity in MT regrowth assays) with respect to WT cells. These results show that depleting CEP192, PCNT, AKAP450 and CDK5Rap2 only reduced centrosomal MT nucleation by about 50%. As far as we know, the conditions to inhibit MT nucleation at the centrosome has not been settled yet. CEP192 has been reported to displace NEDD1 from the centrosome (Zhu et al., CB 2008). So, our results might also apply for NEDD1.

Minor Comments:

-The use if the abbreviation CTR for centrosome, makes the text unnecessarily confusing. Modified
-Fig. 3A and B, define abbreviations on the gels. Done
-There are many instances where the English is quite awkward. In general, this should be addressed. In particular, the phrase "To better discriminate centrosome and Golgi-associated pools, we previously treated cells with NZ to induce fragmentation and dispersion of the Golgi ribbon..." is hard to understand, but particularly important Do the authors mean that in all experiments looking at Golgi MT growth, cells were pre-treated with NZ to induce Golgi fragmentation?
Yes, we do mean that, as can be seen in Figs. 4 and 5. The main reason for proceeding in this way is that the centrosome is usually surrounded by the GA. This makes difficult to distinguish between centrosome- and Golgi- associated pools of proteins that localize to both organelles. The most widely used way to dissociate both organelles is indeed to induce Golgi fragmentation and dispersion by perturbing MT network.

Referee #3:

The manuscript describes the knockout of the three centrosome proteins pericentrin, AKAP450, Cdk5rap2, and double-KO combinations of pericentrin/AKAP450 and pericentrin/Cdk5rap2, in human hTERT-RPE1 cells. All three centrosome proteins are known to bind gamma-tubulin complexes. Several unexpected observations are reported from these KO-experiments: double-KO of pericentrin and AKAP450 reduces drastically the protein levels of Cdk5rap2, and causes complete absence of Cdk5rap2 from the centrosome. Despite the simultaneous loss of three gamma-tubulin-interactors, the centrosomal recruitment of gamma-tubulin and the capacity to nucleate microtubules from the centrosome remain nearly unaffected. In contrast, microtubule nucleation from Golgi membranes needs at least the protein AKAP450, confirming previously published data. Elimination of centrosomes by centrinone-treatment promotes the recruitment of centrosome proteins to Golgi membranes and increases their capacity of microtubule nucleation, whereas centrosome amplification inhibits nucleation from Golgi elements. In the absence of centrosomes, knockout of AKAP450 suppresses microtubule nucleation from the Golgi apparatus, but still enables the formation of a cytoplasmic microtubule network. Silencing of pericentrin in these centrinone-treated AKAP-KO cells delays microtubule regrowth, but does not block it. Finally, it is claimed that the absence of centrosomes increases the amount of microtubule polymer and the density of microtubules in cells.

Major points:
1) In my opinion, a lot of interesting points are raised in this study, but the present manuscript lacks a clear and novel message. Part of the data may simply be explained by a competition for microtubule-nucleating/organizing proteins between the centrosome and non-centrosomal sites. Although the major conclusion of this manuscript is original (i.e. centrosomes modulating microtubule nucleation from non-centrosomal sites by toning down their activity and by acting as sensors for microtubule mass), this point is poorly supported by experiments.
Changes in microtubule mass, dependent on the presence or absence of centrosomes, are only assayed photometrically, by quantification of microtubule immunofluorescence. I think that this assay may not be sufficient to support the authors' claim. In particular, the amount of microtubule polymer in centrosome-containing cells should be too high near the centrosomal microtubule-organizing centre to allow correct measurements (problem of saturation and resolution of immunofluorescence signal), leading to an underestimation of polymer mass.
We do not think so for several reasons. First, we tried to minimize saturation during image acquisition. Second, differences in MT mass polymer are huge (5 times more in centrosome-less cells than in control cells). Underestimation of centrosomal signal due to saturation can hardly explain such big variations. Thirdly, similar values were obtained when EB1 comets were quantified instead of MT mass polymer and, in this case, there are no problems of saturation. Finally, in cells containing several centrosomes by washing out centrinone (see Fig. 7), no differences in MT density were detected although more problems of saturation and resolution should take place.

An independent method, such as microtubule pelleting from centrinone-treated and untreated cells should be used to verify the immunofluorescence data.

We are not sure that in vitro MT pelleting could reproduce the effect of centrosome loss in MT organization. MT pelleting is usually performed from the cytosolic fraction after eliminating nuclei, centrosomes and most of the microsomal fraction by centrifugation. MTs are then induced to polymerize by warming and finally sedimented through a sucrose cushion. Protein concentration of cytosolic fractions should be normalized in order to compare the results (in our experience protein concentrations higher than 3 mg/ml produce artefactual aggregation of tubulin rather than MT polymerization). In addition, since the centrosome and a large fraction of Golgi membranes are eliminated from cytosolic fractions, it would be difficult to make sure that the *in vivo* mechanisms are reproduced in the *in vitro* pelleting experiments.
Following referee' 2 suggestion, we have measured a- and b-tubulin levels of control and centrinone-treated cells by WB and normalized the values with respect to either Hsp70 content or total protein level (Fig. EV7). No substantial differences were detected in any case as shown in Fig. EV7.

2) The data on non-centrosomal microtubule nucleation are descriptive, but very limited novel mechanistic information is provided: the importance of AKAP450 for microtubule nucleation from the Golgi apparatus has already been published. On the other hand, how cytoplasmic microtubules are formed in the absence of centrosomal and Golgi-dependent organization remains largely unclear (obviously, pericentrin is not essential, since its absence only causes a slight delay in cytoplasmic microtubule re-growth).
We cannot agree with this interpretation, nor does referee 1 actually. We have now included a more detailed description of the PCNT-independent cytoplasmic MT nucleation. Our results indicate that MTs grow as single units, in contrast with MT growing as mini-asters from PCNT-containing cMTOCs. Therefore, the absence of PCNT does not only causes a delay in cytoplasmic MT regrowth but induces a different mode of cytoplasmic nucleation. In addition, we have treated centrosome-free *akap* KO cells depleted or not of PCNT with the g-tubulin inhibitor gatastatin that has been reported to partially inhibit and delay MT nucleation (Chien et al., Nat Comm 2015). Our results demonstrate that g-tubulin is responsible for both PCNT-dependent and independent cytoplasmic MT nucleation.
In summary, we show in the new version that PCNT-independent cytoplasmic microtubule nucleation is a g-tubulin dependent process able to generate cell-wide MT networks from single MTs.

A testable hypothesis would be augmin-dependent nucleation of "secondary" microtubules, if a minimum of "primary" microtubules were formed.
Although this possibility cannot be excluded, the absence of branched MTs in our MT regrowth experiments argue against it. Following referee 1 suggestion we have investigated the role of g-tubulin in this process.

3) It remains largely unexplained by what mechanism gamma-tubulin is still recruited to the centrosome, in the absence of AKAP450, pericentrin, and Cdk5rap2.
We show in the new version that CEP192 is responsible for 44.4% of centrosomal g-tubulin recruitment in wild-type cells. In CEP192-depleted double PCNT-AKAP450 KO cells this percentage increased until 53.5% (Fig 3H). There must be additional mechanisms to g-tubulin recruitment to the centrosome.

Minor points:
- Page 13, line 436: I think the statement in this line is oversimplified; the presence of chTOG and TPX2 may have conformational effects on tubulin that support polymerization, independent of tubulin concentration (for example, see Zhang et al., 2017, Elife Nov 9;6. pii: e30959). Modified in the new version
- Page 9, line 284, the wording is unclear: "levels ... significantly increased in centrinone-treated cells (151% and 154% increase, respectively)" - you mean an increase from 100% to 151% and 154%...? We have clarified this point.
- Do the symbols in Figure S4D represent data from Figure 7 C-F? Yes, they do.

2nd Editorial Decision 22 June 2018

Thank you for the submission of your revised manuscript to EMBO reports. I apologize again for my delayed response but we have only recently received the last referee report and I am currently traveling. Please find the full set of referee reports copied below.

As you will see, all referees acknowledge that the manuscript has been significantly strengthened during the revision and support publication in EMBO reports after the addition of some additional control experiments and some further clarification of text and figures. Please address the remaining referee concerns and please also provide a point-by-point response upon resubmission.

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

- Please reformat references according to the style of EMBO reports. The first 10 authors are listed followed by et al. You can download the respective EndNote file from our Guide to Authors (https://drive.google.com/file/d/0BxFM9n2lEE5oOHM4d2xEbmpxN2c/view)

- Please note that all new results in the Abstract should be reported in present tense. Please also note that the Abstract should not exceed 175 words.

- Please note that we can only accommodate up to 5 EV figures. Therefore, please move two of the figures to an Appendix (maybe EV1 and EV2?). The Appendix is a single pdf file with 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

- Please provide a completed Author Checklist. The Checklist will be published alongside your article. You can download it from our Author Guidelines webpage.

- Please provide a header for the Conflict of Interest paragraph.

- Statistics: We noticed that many statistical calculations are based on two independent experiments. Please note that the application of statistics is not advisable if the number of independent experiments is smaller than two, even if each sample comprises many different cells. I would therefore suggest removing the p-values from these panels. I also made some more comments in the attached Word file.

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large. For the larger image the height is variable. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS
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Referee #1 (former Referee #3):

I acknowledge that the authors have made a considerable effort to improve their manuscript.
In particular, the new experiments on CEP192 represent a valuable addition.
However, I still think that their argument that microtubule mass is modulated by the presence or absence of centrosomes (a major point of this manuscript, and a very original one, indeed!) should be backed up by an independent (biochemical) method. I may have been imprecise in my previous review on how to conduct such a control experiment: I think it should be feasible to lyse cells under microtubule-stabilizing conditions (PIPES, Mg2+, EGTA, GTP, eventually glycerol) and to

determine by immunoblotting the presence of tubulin in the soluble and insoluble fractions. This should provide a rough estimate of the "microtubule mass" (that should be restricted to the insoluble fraction), and whether it varies dependent on the presence of centrosomes.

Referee #2 (former Referee #1):

I am basically satisfied with the revised manuscript and in favour of publication. However, some typographical and grammatical errors should be corrected prior to publication, e.g. Line 47 [MZT1], L62-63, L492). In addition, it appears that the authors misinterpreted my previous comment-3, where I intended to suggest the addition of a control immunoprecipitation using cell extracts derived from the KO line (AKAP450 KO line for Fig. 3A, and PCNT KO line for Fig. 3B).

Referee #3 (former Referee #2):

This revised manuscript by Gavilan et al. explores the relationship between different MTOCs, in particular the centrosome and the Golgi in RPE1 cells. The authors propose a hierarchy of MTOC function: centrosome > Golgi > cMTOCs > cytoplasmic microtubules. Overall, the authors have addressed the majority of my concerns or changed the language where appropriate. However, the text is still fraught with issues that will need to be addressed:

Line 215: As I raised in my original review, I still take issue with the simple model "that PCNT and AKAP450 compete for PACT-domain binding sites at the PCM." If this was the case, overexpression of PACT should affect the localization of AKAP450 or PCNT, which the authors argue is not the case.

Line 140-141: Thus, the stability of CDK5Rap2 appears to depend on its interaction with AKAP450 and /or PCNT. Without looking at mRNA expression, you cannot conclude this is an effect on protein stability.

Line 153: "no major differences between WT and AKAP450 depleted cells were observed", yet the figure shows a significant change (albeit small) denoted by an asterisk.

Figure 3H- why are there two g-tubulin foci in 2/3 panels?

Figure 4E-% of cell with GA nucleation-why not measure MTs/GA fragment as above? Is this a qualitative assessment of whether there are or are not MTs associated with each GA fragment?

Figure 5E- needs quantification or at very least n values.

Line 382-383: Emerging microtubules were identified by EB1 labeling. However, Figure 6 and most of EV6 show a-tubulin labeling.

The writing is still very awkward in a number of places, making the paper difficult to read. In. general, the text could be edited for clarity.
For example:

The authors continually refer to centrosome instead of 'the centrosome' or 'centrosomes'. This should be fixed.

Line 47: MZT1 not MTZ1

Line 191: instead, more dependent [on] CEP192 and [on] additional uncharacterized mechanisms.

Line 224: by 21%

Line 318: conditions, was [localized] to the GA

Line 458: were still able [to]

2nd Revision - authors' response 13 July 2018

Enclosed the final version of our manuscript ref EMBOR-2018-45942V2. We have addressed all the referee's comments as you can see in the point-by point response below. The manuscript has also been edited by a professional English Editor, as suggested by one of the referees.

More importantly, in order to apply statistics to our results we have added data from a third experiment in all quantifications of Figures 2, 3 (except E and I) and 4. Some data and p values have slightly changed after adding the new results reinforcing our conclusions.

We have also made all the editorial modifications you required and included the synopsis and the synopsis graph.

Point-by-point response

Referee #1 (*we assume that new referee #1 is previous referee 3*)

I acknowledge that the authors have made a considerable effort to improve their manuscript. In particular, the new experiments on CEP192 represent a valuable addition.
However, I still think that their argument that microtubule mass is modulated by the presence or absence of centrosomes (a major point of this manuscript, and a very original one, indeed!) should be backed up by an independent (biochemical) method. I may have been imprecise in my previous review on how to conduct such a control experiment: I think it should be feasible to lyse cells under microtubule-stabilizing conditions (PIPES, Mg2+, EGTA, GTP, eventually glycerol) and to determine by immunoblotting the presence of tubulin in the soluble and insoluble fractions. This should provide a rough estimate of the "microtubule mass" (that should be restricted to the insoluble fraction), and whether it varies dependent on the presence of centrosomes.

In the first round of revision, referee #3 suggested MT pelleting experiments from centrinone-treated and untreated cells as an independent method to verify the immunofluorescence data. In our previous point-by-point response we argued that *in vitro* MT pelleting experiments could hardly reproduce the effect of centrosome loss in MT organization in cells.
We interpret that he/she is now proposing *in situ* lysis of centrinone-treated and untreated cells in a MT-stabilizing buffer. So, we have performed these experiments and compared by WB the distribution of tubulin between soluble and insoluble fractions. Data obtained from three independent experiments revealed that the ratio between soluble and insoluble tubulin was 1.19 in WT cells whereas it was 0.89 in centrinone-treated cells. Although these results suggest that centrinone-treated cells contain more MTs than WT cell, the standard deviation of data from different experiments was quite high and no clear conclusions could be drawn. Indeed, slight variations of experimental conditions including cell density, temperature, dilution, etc, strongly impacted the results of these experiments. For that reason we decided not to include these data in the manuscript.
It should be noted that at the same confluence the number of cells in centrinone-treated cell dishes is considerably lower than in control cells. In IF experiments shown in Fig. 7 of the manuscript we quantified the alpha-tubulin intensity signal per cell. Centrinone-treated cells are also much more heterogeneous than control cells.

Referee #2 (*we assume that new referee #2 is previous referee 1*)

I am basically satisfied with the revised manuscript and in favour of publication. However, some typographical and grammatical errors should be corrected prior to publication, e.g. Line 47 [MZT1], L62-63, L492). Corrected
In addition, it appears that the authors misinterpreted my previous comment-3, where I intended to suggest the addition of a control immunoprecipitation using cell extracts derived from the KO line (AKAP450 KO line for Fig. 3A, and PCNT KO line for Fig. 3B).

We thought that the referee was referring to the possibility of cross-reaction of antibodies in the western blots following to the co-IPs. Sorry for the misunderstanding.
Anyway, although we did not test the possibility of cross-reaction during co-IPs, results obtained by WBs or IFs suggested that this is not the case. For instance, whether anti-PCNT antibodies cross-

react with AKAP450, one would expect to see a signal by IF in PCNT KO cells when stained with anti-PCNT antibody (and vice versa). Also, AKAP450 and PCNT have similar MW and we did not detect residual bands in western blots that would suggest cross-reaction.

Referee #3 (*we assume that new referee #3 is previous referee 2*)

This revised manuscript by Gavilan et al. explores the relationship between different MTOCs, in particular the centrosome and the Golgi in RPE1 cells. The authors propose a hierarchy of MTOC function: centrosome > Golgi > cMTOCs > cytoplasmic microtubules. Overall, the authors have addressed the majority of my concerns or changed the language where appropriate. However, the text is still fraught with issues that will need to be addressed:

Line 215: As I raised in my original review, I still take issue with the simple model "that PCNT and AKAP450 compete for PACT-domain binding sites at the PCM." If this was the case, overexpression of PACT should affect the localization of AKAP450 or PCNT, which the authors argue is not the case. We have now modified the sentence.

Line 140-141: Thus, the stability of CDK5Rap2 appears to depend on its interaction with AKAP450 and /or PCNT. Without looking at mRNA expression, you cannot conclude this is an effect on protein stability. We agree. We have now modified the sentence.

Line 153: "no major differences between WT and AKAP450 depleted cells were observed", yet the figure shows a significant change (albeit small) denoted by an asterisk. We have modulated the sentence.

Figure 3H- why are there two g-tubulin foci in 2/3 panels?
There is no special reason. In our experience, in an asynchronous cell culture centrioles are slightly separated in a percentage of cells.

Figure 4E-% of cell with GA nucleation-why not measure MTs/GA fragment as above? Is this a qualitative assessment of whether there are or are not MTs associated with each GA fragment?
Yes, it is. Since the presence of a high number of centrosomes abolished MT nucleation from the GA, it made more sense, in our opinion, to determine the number of cells that have lost this capacity than to measure the number of MTs per Golgi fragment that was zero in many of these cells.

Figure 5E- needs quantification or at very least n values.
As for 4E, these are qualitative estimations of the presence or absence of either PCNT or CDK5Rap2 at the GA in KO cell lines treated with centrinone. In cells lacking centrosomes and AKAP450, both PCNT and CDK5Rap2 were fully displaced from Golgi membranes. These results demonstrated that the association of both proteins to Golgi membranes in centrosome-less cells depends on AKAP450. In our opinion, this conclusion does not require quantification. I would like to note that this concern was not raised in the first round of revision.

Line 382-383: Emerging microtubules were identified by EB1 labeling. However, Figure 6 and most of EV6 show a-tubulin labeling.
But in Fig 2I, 4A, 4D and 6B emerging microtubules were labeled with EB1. We chose EB1 or alpha-tubulin depending on the other antibodies when doing double and triple labeling.

The writing is still very awkward in a number of places, making the paper difficult to read. In. general, the text could be edited for clarity. The manuscript has now been edited by a professional editor.
For example:
The authors continually refer to centrosome instead of 'the centrosome' or 'centrosomes'. This should be fixed. Corrected

Line 47: MZT1 not MTZ1. Corrected.

Line 191: instead, more dependent [on] CEP192 and [on] additional uncharacterized mechanisms. Corrected
Line 224: by 21% . Corrected
Line 318: conditions, was [localized] to the GA. Corrected
Line 458: were still able [to]. Corrected

3rd Editorial Decision 21 August 2018

Thank you for the submission of your revised manuscript to EMBO reports. It had been sent back to referee 1 for an evaluation of the biochemical assay to quantify microtubule polymer mass. As you will see from the report below, this referee questioned the robustness of the finding that microtubule mass is modulated by the absence of centrosomes since it could not be detected when the cells were lysed and tubulin was quantified in the insoluble fraction. I have subsequently asked referee 3 to evaluate the final version of your manuscript and to comment on this remaining concern from referee 1. As you will see from the report below, also referee 3 was concerned that the biochemical method failed to detect the increase in microtubule polymer mass observed using immunostaining techniques. In order to make a fair and informed decision on this manuscript, I further consulted an editorial advisor with expertise in centrosome and microtubule biology whose opinion we trust. I have explained the concerns of the referees regarding the data shown in Figure 7 and I also detailed your response to the referee concerns. The advisor replied and supported the validity of the chosen approach, i.e., to fix and stain the cells and to quantify fluorescent intensity. The advisor noted that "… if you had asked me how to quantify microtubule density in a cell under different conditions, I would have suggested to fix and stain the cells and quantify the fluorescent intensity." The advisor also indicated that polymer levels can indeed be affected by a variety of factors during cell lysis. S/he noted: "I also would have strongly suggested against a biochemical strategy to test this particular question. For a biochemical strategy, when you lyse the cells, there are going to be a wide range of factors that affect polymer levels (buffer, lysis conditions, etc) and it would have very little to do with the actual nucleators in a cell." Overall, the advisor supported publication of the current dataset and noted: "I feel much more comfortable with them being able to test the assembled polymer that exists (not what occurs following biochemical perturbation) and from being able to analyze this feature in individual cells focusing particularly on those cells that display the relevant phenotype. I consider this to be an accepted method in the field, and also one that I am confident with in this case.". Given this positive evaluation, we have decided to proceed with the publication of your manuscript.

Please address the remaining concerns of referee 3 in the manuscript and please also provide a complete point-by-point response.

Moreover, I appreciate that data from a third experiment was added in several cases. Yet, I notice that the number of experiments did apparently not change for Fig. 3E and 3I and this should be indicated in the figure legend. Currently the Data information states that the data were collected from three independent experiments. You could include the phrase "except for E and I, which are based on two independent experiments" and remove statistics from 3I.

We routinely compare the provided source data with the figure panels. Doing so I noticed a small mistake in the source data for Figure EV3. It is labeled with "A" while the corresponding figure panel is in "B".

I am looking forward to receive the final version of your manuscript.

REFEREE REPORTS
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Referee #1:

The authors claim that centrosome loss increases microtubule numbers, since they detect more than five-fold increase in "microtubule mass" after centrinone treatment, based on photometric analysis. In my opinion, this is an error-prone analysis, since neither cell volume is taken into account, nor problems of saturation of fluorescence signal, nor variation in immunostaining dependent on cell density. The more relevant experiment, i.e. comparison of soluble tubulin pools and insoluble tubulin pools (the latter representing microtubule polymer) of centrinone-treated and control cells reveals no significant difference. If the centrinone-induced five-fold increase (!) in polymer mass were correct, the biochemical experiment should pick up some difference. In my opinion, the ensemble of experiments do not support the authors' conclusion.
Arguments are made in the rebuttal letter, questioning the relevance of the biochemical approach, since the outcome may vary dependent on cell density etc.. I disagree with this, since the

biochemical data rely on cell numbers far larger than those analyzed photometrically. Moreover, one should acknowledge that the same arguments can be made to question the validity of the photometric data in Figure 7!

Referee #3:

I have some reservations with the newest revision submitted by Gavilan et al. The authors have added new data during this round of revision that makes me a bit concerned with their conclusion that microtubule polymer mass is increased in cells lacking centrosomes. They had presented this conclusion based on quantification on antibody staining, but are now unable to substantiate this claim by comparing amounts of sedimented microtubule polymers to free tubulin from DMSO and centrinone treated- cells. I had raised concerns in the original submission that quantification of immunostaining does not truly reflect microtubule polymer as they are assessing total tubulin when they do this. These new results are a bit alarming given the large difference they are able to detect by immunostaining and take some of the wiond from the sails of one of the major conclusions of the paper.

In addition, the authors did little in the way of addressing the small concerns I had raised in the last review cycle.

Original statement: "Line 215: As I raised in my original review, I still take issue with the simple model "that PCNT and AKAP450 compete for PACT-domain binding sites at the PCM." If this was the case, overexpression of PACT should affect the localization of AKAP450 or PCNT, which the authors argue is not the case."

This sentence still appears in the text at line 206-207. How is this still a plausible model?

Original statement: "Figure 5E- needs quantification or at very least n values."

Authors' response: "As for 4E, these are qualitative estimations of the presence or absence of either PCNT or CDK5Rap2 at the GA in KO cell lines treated with centrinone. In cells lacking centrosomes and AKAP450, both PCNT and CDK5Rap2 were fully displaced from Golgi membranes. These results demonstrated that the association of both proteins to Golgi membranes in centrosome-less cells depends on AKAP450. In our opinion, this conclusion does not require quantification. I would like to note that this concern was not raised in the first round of revision."

New response: Would it be terribly difficult to give n values so I know that you are not showing the only field of cells you looked at? Also, I was under the impression that this is a new round of review, so it shouldn't matter if I raised this point in the first submission or not.

Original statement: "Line 382-383: Emerging microtubules were identified by EB1 labeling. However, Figure 6 and most of EV6 show a-tubulin labeling."

Authors response: "But in Fig 2I, 4A, 4D and 6B emerging microtubules were labeled with EB1. We chose EB1 or alpha-tubulin depending on the other antibodies when doing double and triple labeling."

New response: This statement is in a paragraph where Figure 6 is being discussed. Almost all panels in Figure 6 use a-tubulin staining to look at MTs, thus the above statement is misleading. Would this be so hard to clarify?

3rd Revision - authors' response 27 August 2018

Referee #1:

"The authors claim that centrosome loss increases microtubule numbers, since they detect more than five-fold increase in "microtubule mass"after centrinone treatment, based on photometric analysis".
This is not totally correct. Although we detected this increase in cells without centrioles, we also noted that these cells were bigger than control cells (as Akhmanova's group also noted in Wu et al., 2016). So, MT density, which is the more significant parameter in our opinion, is only twice higher.
"In my opinion, this is an error-prone analysis, since neither cell volume is taken into account (cell volume could not be correlated with microtubule content indeed, but cell surface could), nor problems of saturation of fluorescence signal (note that similar results were obtained when EB1 comet number was quantified as shown in Fig. 7), nor variation in immunostaining dependent on cell density" (we do not understand what this concern means).
"The more relevant experiment, i.e. comparison of soluble tubulin pools and insoluble tubulin pools (the latter representing microtubule polymer) of centrinone-treated and control cells reveals no significant difference". If the centrinone-induced five-fold increase (!) in polymer mass were correct (we can provide all the photos we used to make this quantification, if necessary) the biochemical experiment should pick up some difference. In my opinion, the ensemble of experiments do not support the authors' conclusion. Arguments are made in the rebuttal letter, questioning the relevance of the biochemical approach, since the outcome may vary dependent on cell density etc… I disagree with this, since the biochemical data rely on cell numbers far larger than those analyzed photometrically.
This is a valid argument only when dealing with an homogeneous population of cells. Which is not the case here. In our previous rebuttal letter we claimed that after several days of centrinone treatment we detected a number of cells that still contained a centriole, an increase in apoptotic cell death and numerous mitotic errors. These phenotypes had been previously reported by Wong et al., Science, 2015. We also noted some cell adhesion problems.
We still think that biochemical quantification of MT mass is not the best method since MT stability is very sensitive to experimental conditions (temperature, time, cell density, etc). Even more, it is known that MTs are quickly depolymerized by dilution what happens when cells are extracted with detergent-containing buffers.

Referee #3:

I have some reservations with the newest revision submitted by Gavilan et al. The authors have added new data during this round of revision that makes me a bit concerned with their conclusion that microtubule polymer mass is increased in cells lacking centrosomes. They had presented this conclusion based on quantification on antibody staining, but are now unable to substantiate this claim by comparing amounts of sedimented microtubule polymers to free tubulin from DMSO and centrinone treated-cells.
As we mentioned in our previous rebuttal letter, we think that quantification of MT mass by WB of soluble and insoluble fractions is not a good method. It is known that MT stability is very sensitive to experimental conditions (temperature, time, cell density, etc). In addition, MTs are quickly depolymerized by dilution what happens when cells are extracted with detergent-containing buffers.
Finally, centrinone-treated cell culture is very heterogeneous. After several days of centrinone treatment we detected a number of cells that still contained centrioles, an increase in apoptotic cell death and numerous nuclear perturbations. These phenotypes had been previously reported by Wong et al., Science, 2015. We also noted some cell adhesion problems. For those reasons, a photometrical analysis seemed to us a more precise way to determine MT mass.

I had raised concerns in the original submission that quantification of immunostaining does not truly reflect microtubule polymer as they are assessing total tubulin when they do this.
In the first revision this referee asked for determining the concentration of tubulin in control and centrinone-treated cells. Quote : "*based on the data, it is plausible to conclude that centrinone treatment increase tubulin expression, which would itself lead to an increase in microtubule nucleation at different MTOCs. It is important to understand whether the loss of the centrosome per se or whether a total increase in tubulin levels is responsible to the general increase in microtubule nucleation from the Golgi*". So, his/her concern in his/her previous revision was about the mechanism regulating MT number nor about the method to quantify it. Even more, he/she did not mention this point in his/her second revision and was apparently satisfied with our experiments on alpha- and beta-tubulin concentration.
These new results are a bit alarming given the large difference they are able to detect by immunostaining and take some of the wind from the sails of one of the major conclusions of the paper.
In addition, the authors did little in the way of addressing the small concerns I had raised in the last review cycle.
Original statement: "Line 215: As I raised in my original review, I still take issue with the simple model "that PCNT and AKAP450 compete for PACT-domain binding sites at the PCM." If this was the case, overexpression of PACT should affect the localization of AKAP450 or PCNT, which the authors argue is not the case."

This sentence still appears in the text at line 206-207. How is this still a plausible model?
In the second version, this sentence was modulated following the indications of the referee: “one possibility is that PCNT and AKAP450 compete for PACT-domain binding sites at the PCM”. In our view, the fact that the overexpression of a tagged version of the PACT domain (whose properties might be different to those of the same domain in the context of the full-length native protein) do not displace endogenous PCNT or AKAP450, does not completely rule out the possibility that these protein can compete for common binding sites at the PCM. We only propose this mechanism as “one possibility”.

Original statement: "Figure 5E- needs quantification or at very least n values."
Authors' response: "As for 4E, these are qualitative estimations of the presence or absence of either PCNT or CDK5Rap2 at the GA in KO cell lines treated with centrinone. In cells lacking centrosomes and AKAP450, both PCNT and CDK5Rap2 were fully displaced from Golgi membranes. These results demonstrated that the association of both proteins to Golgi membranes in centrosome-less cells depends on AKAP450. In our opinion, this conclusion does not require quantification. I would like to note that this concern was not raised in the first round of revision."
New response: Would it be terribly difficult to give n values so I know that you are not showing the only field of cells you looked at?
It is just the contrary. We performed this experiment, very simple indeed (IF of KO cells treated with centrinone), several times. Once again, since the phenotype was very clear (presence/absence) we considered unnecessary to quantify this experiment (we did not envisage the possibility that someone could think that images we were showing corresponded “to the only field of cells we looked at”). Anyway, we have included n=3 in the final version since we took pictures from three different experiments.
Also, I was under the impression that this is a new round of review, so it shouldn't matter if I raised this point in the first submission or not.

Original statement: "Line 382-383: Emerging microtubules were identified by EB1 labeling. However, Figure 6 and most of EV6 show a-tubulin labeling."
Authors response: "But in Fig 2I, 4A, 4D and 6B emerging microtubules were labeled with EB1. We chose EB1 or alpha-tubulin depending on the other antibodies when doing double and triple labeling."
New response: This statement is in a paragraph where Figure 6 is being discussed. Almost all panels in Figure 6 use a-tubulin staining to look at MTs, thus the above statement is misleading. Would this be so hard to clarify?
In our hands, at very short NZ-washout time points MT labelings displayed by either anti-a-tubulin or anti-EB1 antibodies were almost identical. So, we decided to use one or another antibody based on the compatibility with the other antibodies when doing double or triple labelings. Sometimes, however, EB1 labeling appeared more suitable due to the lack of background that is more prominent when using an anti-a-tubulin antibody. This is the case of gatastatin experiments shown in Fig. 6G.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Rosa M. Rios
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-45942V2

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 statistical method was used to predetermine sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	In scatter or box-and-whisker plots, only values between 10th and 90th percentiles were displayed and analyzed.
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.	Cell culture dishes were randomly selected for treatments.
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	Pictures used to quantifiy phenotypes were taken randomly by different investigators.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Significant differences among groups were evaluated by unpaired two-tailed Student's t-test (two samples) or one-way ANOVA (more than two samples) and indicated when relevant.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, in almost all cases. D´Agostino-Pearson or Kolmogorov-Smirnov normallity tests were applied.
Is there an estimate of variation within each group of data?	Yes, using standard deviations or displaying all values between 10th and 90th percentiles.

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Is the variance similar between the groups that are being statistically compared?	Yes, in the vast majority of cases.

C- Reagents

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).	Yes. See Materials and Methods (Cell culture, antibodies and treatments).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Immortalized human pigment epithelial cells hTERT-RPE1 were purchased from ATCC (year 2009). hTERT-RPE1 FRT/TO cells were provided by J Pines in 2011 (Gurdon Institute, Cambridge, UK). Mycoplasma contamination is periodically checked by PCR and DNA stainning (DAPI). Cell line authentication was not done.

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	NA
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).	Source data for all immunoblots is available in Expanded View section.
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).	NA
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.	NA

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