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RBM14 prevents assembly of centriolar protein complexes and maintains mitotic spindle integrity

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(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

21 May 2014

Thank you for submitting your manuscript to The EMBO Journal. We have now considered it within our editorial team, and unfortunately come to the conclusion that we cannot offer publication in The EMBO Journal. We appreciate the rationale of your attempt to identify new regulators controlling the number of newly formed centrioles, and realize that your observations on induction of ectopic centriolar-like protein complexes upon RBM14 knockdown are potentially interesting. However, we are not convinced that your follow-up experiments describing an apparently non-canonical amplification process, while likely of interest to others in the field, provide sufficiently definitive understanding of this process nor sufficiently conclusive insights into the molecular roles of RBM14 in it. Moreover, we feel that the exact regulatory role and context of RBM14 centriole-related functions in relevant physiological or pathophysiological settings remains to be established beyond the level of knock-down phenotypes. As it stands, I am afraid we have to consider the study at the present stage still too preliminary and descriptive to constitute a compelling advance warranting publication in a broad general title such as The EMBO Journal, and therefore currently better suited for a somewhat more dedicated cell biology journal. I am sorry to have to disappoint you on this occasion but nevertheless hope that this negative decision will not discourage you from considering The EMBO Journal for publication of future studies.

Thank you for your message in which you indicated that you are against sending for in-depth review our manuscript entitled "RBM14 prevents de novo assembly of centriolar protein complexes and maintains mitotic spindle integrity".

I am writing because I am very puzzled by the nature of your concern and also why such concern would lead you to reject the manuscript.

In despite of accumulating observations that de novo centriole formation seems to be pathophysiologically important to understand a cause of genome instability in tumorigenesis, prior to our study, the way of de novo formation of centrioles/centriole-like structures which are frequently found in some types of cancer cells was unclear. Our work is the first demonstration that de novo formation of centrioles occurs even in the presence of pre-existing centrioles and leads to significant defects in formation of mitotic spindles and chromosome segregation in a variety of cancer cells. Our substantial/compelling data clearly indicate the detailed molecular basis that RBM14 normally suppresses such de novo centriole formation by regulating the function of core centriole proteins, the STIL/CPAP complex and HsSAS-6. We still believe that the findings shown in the manuscript will be of broad significance in cell and cancer biology and thus suited for publication in the EMBO journal.

We further establish that centrioles can assemble de novo in a retrograde fashion. Centriole formation normally starts with the assembly of a cartwheel structure that serves as a scaffold, but what we found in this study is that the precursors composed of centriole middle-distal parts first gather in the cytoplasm and in turn incorporate the cartwheel structure in a retrograde fashion to form complete centrioles de novo. As you can see in the manuscript, this conclusion is supported by substantial amount of compelling data including thorough cell biology and biochemistry, high-resolution live imaging and sophisticated Correlative Light-Electron Microscopy (CLEM).

Furthermore, our study clearly demonstrated the detailed/direct molecular mechanisms how a tumor suppressor RBM14 depletion leads to such de novo formation of centrioles. We find that RBM14 physically interacts with the core centriole protein STIL and interferes with a protein complex formation of STIL and CPAP, thus normally preventing formation of aberrant centrioles. However, when RBM14 depleted, the STIL/CPAP complex assembles other centriolar components and pericentriolar materials on its surrounding to form centriolar intermediates in the cytoplasm. These structures thereafter incorporate HsSAS-6, a core cartwheel component to become complete/functional centrioles. Our sophisticated time-lapse imaging also provides the direct evidence that the de novo-formed centrioles can form ectopic mitotic spindles that induce significant chromosome segregation error, leading to genome instability.

Please note that this manuscript was also sent for in-depth review in the Science journal. Although the reviewers expressed potential interest on our study, one reviewer claimed the lack of structural analysis on the de novo-formed centrioles that we found. However, we conducted detailed structural analysis on the structures by using Correlative Light-Electron Microscopy (CLEM), which clearly provides the structural insights on the way of de novo formation of centrioles. Thus, our manuscript was further refined and we still remain of the opinion that our manuscript will be of great interest for the readership of the EMBO journal.

I would be grateful if you could consider the above arguments, discuss it with the other editors if need be, and contact me thereafter at your earliest convenience

Thank you for your patience while I was looking into your manuscript once more, and discussing your arguments not only with my colleagues but also with an external advisor who is an expert in this field. Clearly, some of our original reservations regarding the unclear molecular role of RBM14 in centriole formation, and its unclear physiological regulatory function, remain valid and are not readily addressed by the points in your response. On the other hand, we realize that your

observations on de novo centriole formation in the presence of pre-existing ones and in an apparent retrograde fashion is potentially of more general significance to the field, and would therefore be willing to give the manuscript the benefit of the doubt by sending it to external reviewers. I should however stress that we will really require strong and enthusiastic support from the referees in this case, who - as stressed by our expert advisor - will need to be convinced by your CLEM data that RBM14 knockdown really induces formation of genuine centrioles rather than to some artificial microtubule-organizing structures more frequently observed in tissue culture cells such as those used here.

I shall be in contact again as soon as we will have heard back from the referees.

2nd Editorial Decision

25 June 2014

We have now received the reports from three expert referees who had agreed to review your study on RBM14 prevention of de novo centriole-like structure formation. All three referees consider this work in principle interesting and of potential importance, even in the absence of further insights into RBM14 physiological roles and regulation. However, they do raise a number of serious concerns regarding both the understanding of the mechanism of RBM14 action on the molecular level, and the molecular nature and structure of the observed centriole-like complexes; thus confirming some of the original concerns from the editors and the external editorial advisor. In our view, these combined reservations currently preclude publication of your manuscript in The EMBO Journal.

Given the overall interest expressed by the referees, and the fact that all referees offer a number of constructive suggestions for improvement of this work, I would nevertheless remain open to considering a revised version of the manuscript further for publication, in case that you should be prepared to substantially strengthen and extend the study. One key aspect to further address would be the mechanistic role of RBM14, including its endogenous localization, complex formation/disruption, or exclusion of gene expression roles in this case (see in particular points 1, 3, 4, 5, 7 of referee 1, and points 3, 4, 5 of referee 2). The other essential point would be better insight into the ultrastructure and assembly of the observed centriolar protein complexes and centriole-like structures (especially points 9, 10 of ref 1, points 1, 2 of ref 2, and points 1, 2, 3 of ref 3). On the other hand, I do not feel that ref 1 pt 6 and ref 3 pt 5 would need to be experimentally addressed within the scope of the present study.

I realize that adequately addressing these key points may not be trivial and will likely require a substantial further amount of time and experimental effort, and I would therefore also understand if you preferred to rather seek rapid publication without major changes elsewhere. However, in light of the well-taken concerns raised by our referees I do agree that only these requested improvements and extensions would make this study a more compelling candidate for publication in The EMBO Journal, and eventual acceptance of the paper would be dependent on satisfactorily addressing these essential issues, as well as adequately answering to the other, more specific points raised. Since it is our policy to allow only a single round of major revision to achieve this, I would be open to discussing an extension of our standard three months revision time up to a maximum of six months here, in case you should decide to submit a revised version to our journal. Please note that during such an official revision period, competing manuscripts published elsewhere will have no negative impact on our final assessment of your revised study.

Thank you again for the opportunity to consider this work publication. Should you have any further questions regarding this reports or this revision option, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

In this manuscript, Shiratsuchi and colleagues employ mass spectrometry-based proteomic analyses to identify candidate interactors of HsSAS6 and STIL. They conduct small-scale RNAi screening to address their functions in centriole formation in human cells, and identify RBM14, as a putative STIL-interacting protein, and a novel suppressor of de novo assembly of centriole protein complexes. RBM14 can inhibit centriole over-duplication in S-phase arrested U2OS cells, and its depletion induces ectopic centriole protein complexes through interactions with the STIL/CPAP complex. The ectopic centriole protein complexes form in the cytoplasm in a STIL/CPAP-dependent manner and are independent of HsSAS6. They observe that about half the complexes have the ability to nucleate microtubules during mitosis, which leads to pseudo-bipolar spindles and multipolar spindles, and defective chromosome segregation. The authors also conducted RBM14 depletion in mouse embryos and observed the same ectopic centriole protein complex formation. Overall this is an interesting manuscript with a huge amount of work. The results are consistent with the possibility that de novo formation of MTOC can occur independently of cartwheel formation. The biggest weakness of this manuscript is that it tries to accomplish a lot, perhaps too much, and I found it extremely disjointed at times. For example, it begins with a mass spec story on procentriole formation, transitions through de novo centriole assembly, then PCM assembly, mitotic spindle formation and finishes in mouse embryos. Yet it only very partly elucidates how RBM14 actually regulates these processes mechanistically, which to me was the most salient issue. Having said this, if the manuscript is refocused adequately and addresses the following issues, it may become suitable for publication in EMBO.

Major issues:

- 1) The authors need to clarify if endogenous RBM14 localizes to centriole or PCM foci to any reasonable extent. The authors should provide quantitative data using antibodies targeting the endogenous protein and show that the observed labeling goes away after RBM14 RNAi treatment.
- 2) The authors identified RBM14 by mass spectrometry as a putative interactor of STIL and showed it had a function in centriole duplication. It is also mentioned that HsSAS-6 was also analyzed in the same way and that putative interacting proteins were tested by RNAi. I think this data should be included in the manuscript.
- 3) The authors mention a lot of "data not shown" suggesting that the transcriptional activity of RBM14 is not needed for its role in centrosome assembly. I am not sure I totally agree with this argument. In fact, the localization of RBM14 suggests that its activity may lie in the nucleus and the deletion of its RNA-binding domain prevents its ability to rescue the loss of endogenous RBM14. Is there a surgical mutation that can insert in RBM14 that would allow them to further rule-out an indirect transcriptional role? Alternatively, the authors could further substantiate their biochemical data on the RBM14/STIL interaction and the effect of STIL on the STIL/CPAP complex assembly.
- 4) Do the authors know if CPAP/STIL and RBM14 are present in the same protein complex? Are their interactions dependent on other proteins are CPAP, STIL and RBM14 able to assemble independently of other proteins?
- 5) The authors draw the conclusion that cytoplasmic RBM14 could efficiently suppress centriole amplification in S-phase arrested U2OS cells. I feel the foundation upon which this conclusion is based is somewhat weak. They overexpress full-length RBM14 and the C-terminal region of RBM14 (which was suggested to localize to the centrosome, albeit not very efficiently) and found they both suppressed HU-induced centriole amplification. On the other hand, RBM14-PACT, which localizes to the centrosome, did not suppress HU-induced centriole amplification as efficiently as RBM14-NES. From this reviewer's perspective, the two observations seem to contradict each other, and since most of the RBM14-PACT localizes to the nucleus, it is possible that the amount of RBM14-PACT at the centrosome and in the cytoplasm is less than the RBM14-NES, and that's why RBM14-PACT is not as efficient at suppressing centriole over-duplication. Since RBM14 [C] localizes to the centrosome and it is as efficient as RBM14 [FL], I'm just not convinced by their

conclusion.

6) Normal procentrioles are generated in the process of centriole over-duplication in the S-phase arrested U2OS cell assays, which apparently are different from the ectopic centriole protein complexes generated after RBM14 depletion. In this case the authors should address the mechanistic link between normal procentriole formation and ectopic centriole protein complex formation affected by RBM14.

7) The authors should test whether RBM14 overexpression will affect centriole duplication in cycling cells. Also, the authors found that over-expression of RBM14 did not affect formation of multiple procentrioles induced by PLK4, STIL, HsSAS6 overexpression. The difference may be caused by different mechanisms behind centriole overduplication caused by S-phase arrest or PLK4/STIL/SAS6 over-expression in U2OS cells, and it would be good to know where RBM14 is functioning in the process.

8) From my perspective the work on multipolar spindle, chromosome segregation and in vivo mouse is not necessary and does not really increase the impact of the manuscript. I would consider removing it and to focus on the role of RBM14 in centriole/PCM assembly.

9) The authors should clarify if the electron dense structures they observe in absence of RBM14 require PCM components for their assembly.

10) The authors argue that the amorphous structures they observe when depleting RBM14 are somewhat organized like real centrioles (based on distance measurements), yet there are no centrioles. Most problematic here is that they use conventional microscopy to make these claims. I am not sure what point they want to make are the structures they see organized or amorphous or both?

Minor issues:

1) The authors conclude that ectopic centriole protein complex forms at S-phase, because Lovastatin-induced G1-phase arrest inhibited amplification whereas HU-induced S-phase arrest did not. The conclusion can also be supported by observe complex formation of cycling cells accompanied by PCNA staining marking S-phase to rule out artificial affect caused by cell-cycle arrest.

2) The authors suggested that 54% of the centriole protein complexes appeared to harbor microtubule nucleation activity in mitosis. The experiment seems to be done only once and it needs repeating, because it doesn't fit with the observation that only 40% of centriole protein complexes contain α -Tubulin. I'm curious about how the remaining 14% of complexes are able to nucleate microtubules.

3) The authors observed that some small Centrin foci fused with each other and grew to the size comparable with pre-existing centrioles. Is size important for these ectopic complexes to form spindle poles? And how are the foci moving to each other? Is it microtubule dependent? In movie E1/2/3, it seems to me that ectopic centriole protein complexes have a lot of interactions with pre-existing centrioles. It may suggest that centrosomal STIL and CPAP are also responsible for ectopic centriole protein formation.

4) Does RBM14 interact with other centriole duplication and PCM proteins? The authors could use mass spectrometry-based proteomic analysis to identify candidate interactors of RBM14. Alternatively, the authors could confirm the interaction between RBM14/STIL using reciprocal Co-IP experiments and blotting for additional centriole/PCM proteins?

5) The authors repeatedly use words like "interestingly", "strikingly", and "surprisingly" which weighs heavily on the reader... I would try to use them more sporadically and let the readership qualify the observations.

6) The authors mention several times in the manuscript that they will analyze the "molecular architecture". I think this is difficult to do using the methodologies used in the manuscript and the statement needs to be toned down.

7) The authors claim that RBM14 prevents de novo centriole assembly. With the data shown I think this is an overstatement and the title should be changed.

Referee #2:

Centrosomes consist of a pair of centrioles surrounded by the pericentriolar material (PCM) that nucleates and anchors microtubules. Centrioles recruit and organize the PCM. Centriole duplication involves the growth of a new centriole next to a pre-existing centriole and it occurs only once per cell cycle in most proliferating cells. However, in some specialized cells (e.g. multiciliated cells), hundreds of centrioles form almost simultaneously via an acentriolar (de novo) pathway. In this manuscript, Shiratsuchi et al. attempt to persuade the readers that RBM14 acts as a novel suppressor that prevents de novo assembly of "centriolar protein complexes" in proliferating cell cytoplasm. The cytosolic "centriolar protein complex" is a loose term, which has not yet been precisely defined and acknowledged by the Centrosome society. Currently, the composition/structure/function of this complex and its relation to the process of centriole duplication are not clear. Using the mass spectrometry-based proteomic analysis, the authors first identified that RBM14 is a STIL-interacting protein. Interestingly, depletion of RBM14 can induce the formation of ectopic electron-dense amorphous structures in the cytoplasm that possibly contain the "centriolar protein complexes". Further studies showed that the formation of such structures seems not require hSAS-6-containing cartwheel and these structures can function as microtubule organization centers (MTOCs). Intriguingly, a portion of these ectopic "centriolar protein complexes" can also assemble procentriole-like structures containing hSAS-6 in RBM14-depleted cells. Furthermore, they also showed that the interaction of RBM14 with STIL seems to prevent the formation of the STIL/CPAP complex in the cytosol. This would be an interesting finding if confirmed that will provide novel and significant insight into the composition and function of this poorly defined complex. The quality of the data is high and the study nicely combines biochemical work and functional analyses. However, some conclusions lack sufficient experimental support, which needs to be further addressed. The following concerns should be addressed prior publication.

Major points:

- 1) The authors observed that depletion of RBM14 can induce ectopic formation of centriolar protein complexes (Fig. 1). It is also possible that these centriolar protein complexes could be produced from the fragmentation of preexisting centrosomes in siRBM14-treated cells. How do the authors distinguish between these two possibilities?
- 2) To further support the concept that the electron-dense amorphous structures are the sites that contain the centriolar protein complexes in RBM14-depleted cells, the authors need to provide either the CLEM (correlative Light-Electron Microscopy) or immunogold-EM images with antibodies against CPAP and STIL.
- 3) Overexpression of full length RBM14 or RBM14[C] had very minor effect on centriole duplication, while it did dramatically affect centriole amplification in hydroxyurea-treated cells arrested in S phase (Fig. 2). These findings lead the authors to propose that RBM14 may suppress centriole amplification in prolonged S-phase cells. However, endogenous RBM14 (RNA-binding motif protein 14) is mainly detected in the interphase nucleus but weakly in the cytosol by IF analysis (Fig. E2C). To clearly demonstrate that the cytosolic RBM14 could efficiently inhibit centriole duplication in the S-phase cells, the authors should perform Western blot analysis on a sample prepared from both nuclear and cytosolic fractions (not a total lysate) and show that a significant amount of endogenous RBM14 protein does exist in the cytosol.
- 4) CPAP was previously reported to be a STIL-interacting protein. Interestingly, the CPAP-interacting domain in STIL is overlapped with the region binding to RBM14. The authors thus propose that the direct binding of RBM14 and STIL may perturb the function of STIL in centriole duplication possibly via disruption of the CPAP-STIL complex. However, the data presented in Fig. 3E are not strongly enough to support this conclusion. The authors should provide competing IP experiments by transfecting various amount of Flag-RBM14-FL with fixed amount of GFP-CPAP into cells and analyze the ratio of CPAP and STIL with increasing amount of RBM14. Furthermore, the authors should redo the co-IP experiment in Fig. 3A using the lysates prepared from the

cytosolic fraction instead of total cell lysates and examine endogenous RBM14, STIL, and CPAP in the complex with at least two different antibodies (STIL and RBM14) for IP.

5) If the binding of RBM14 to STIL could disrupt the cytosolic CPAP-STIL complex as the authors' proposed, it is reasonable to speculate that ectopically expressed RBM14-FL may also sequester endogenous STIL from the CPAP-STIL complex which resulted in inhibiting centriole duplication. However, it appears not to be the case (Fig. E2B, E4C), why? Furthermore, the authors proposed that the ectopic centriolar protein complexes induced by RBM14 depletion could be the sites for PCM recruitment (Fig. 1E, Fig.E1J) and microtubule nucleation (Fig. 5B). What will happen if RBM14 is overexpressed? Does overexpression of RBM14 inhibit PCM recruitment and MTOC activity?

Minor points:

- 1) Why do the percentages of centrin foci (>4) differ between Fig. 1A (~33%) and Fig. 2A (~18%)? Different siRNA treatments? Please clarify it.
- 2) The authors need to provide statistic SEM in Fig. 7 and Fig. 8
- 3) The Figure 9 legend (hypothetic model) is not clear. The authors need to provide some explanation in the figure legend that links their experiments to when, where and how RBM14 functions on the centriolar protein complexes.

Referee #3:

The work of Shiratsuchi and colleagues, entitled "RBM14 prevents de novo assembly of centriolar protein complexes and maintains mitotic spindle integrity" describes the identification of a novel protein, RBM14, that inhibits the de novo formation of centriole like structures. The authors identified RBM14 as a STIL interacting protein, which is a well characterised centriole duplication protein. RNAi approaches showed that in U2OS RBM14 depleted cells, extra centrosome like structures (centrin, acetylated tub positive structures) were formed. They convincingly ruled out a role of RBM14 in translation that would explain the appearance of these structures. Further work showed that RBM14 prevents the formation of STIL-CPAP complexes and the authors have mapped the domain of interaction. Surprisingly, they show that these centriole-like structures lack Sas-6 and even more surprisingly Plk4 activity. To gain information on the ultra structure level of these structure the authors perform EM analysis and propose that these structures lack a cartwheel and so that they form independently of the canonical centrosome duplication pathway. Nevertheless, these structures act as MTOCs, and if they incorporate Sas-6 they form centriole like structures. They behave as extra centrosomes and in cases of spindle formation these can fail to be bipolar and to correctly segregate chromosomes. Further analysis of mouse embryos depleted of RBM14 confirms in vivo the results obtained in vitro.

This is a very nice paper that identified a novel function for a novel protein in the inhibition of de novo centriole assembly that will be of interest to the centrosome/MT and mitotic fields. Most of the data presented here is of very good quality, contains the adequate controls, quantification and interpretations. I am therefore recommending publication by the EMBO J. However, some of the observations and statements in this version, require extra clarification before publication.

Major points

- 1) The authors convincingly show that RBM14 is involved in the inhibition of the assembly of these centriole like structures in the cytoplasm. However they claim that these structures are not dependent on the presence of Sas-6 or Plk4. I am slightly concerned by these observations. Can the authors provide compelling evidence that Plk4 and Sas-6 have been depleted to lower levels consistent with inhibition of centriole duplication. These proteins (specially Plk4) are low abundant, and so even a reduction might not be sufficient to completely abolish their functionality. So the authors should provide unequivocally evidence that the RBM14 depletion leads to the formation of centriole like structures independently of Sas-6 or Plk4. In Fig E4 they have shown that plk4 RNAi results in the reduction of Sas-6 foci, but are these conditions the same used to analyse the induction of these structures in the absence of RBM14? How about the Sas-6 depletion? Also, for how long were these cells treated for? Is it possible that they are just being arrested prior to S phase? This should be investigated. This is particularly important regarding the identification of RBM14 protein

as a Sas-6 and Stil interactors (First paragraph of results section). How to explain this?

2) Along the same lines the authors show in Fig 4E that these cytoplasmic structures resemble amorphous electron dense material that do not contain sas-6. These figs are extremely difficult to interpret and to distinguish. Can the authors provide figures of better quality? And the quantification of the observed structures. Can also the authors comment of the fact that if these structures are not really bona fide centrioles with a cartwheel, how can they explain that they are able to behave as such since they recruit PCM and nucleate MTs? If these structures are formed independently of Plk4/Sas-6, they only contain STIL and CPAP as core centriole duplication factors? When is centrin incorporated, and Cp110? This should be characterised in a timely defined way.

3) I am not sure that the incorporation of Sas-6 in 8% of these structures is sufficient to state that they become more like centrioles. Here EM is necessary to show that indeed these structures are centrioles- 9 triplets of MTs arranged in a structure that contains a cartwheel .

4) A semantic question. If the structures that form in the cytoplasm are not bona fide centrioles, can the authors used this nomenclature when they describe them. Maybe centriole like?

5) All these experiments were performed in U2OS cells, which are transformed cancer cells. Can the authors deplete RPE1 cells of RBM14 and show whether these structures are present? If it is not the case, it is not a problem but it will be very informative to know if only cancer cell lines can really generate such structures.

Minor points

1) the text clear needs editing. There is too much unnecessary text in the Results section. The authors discuss some of their results in this section and this should be moved to the Discussion section. Also the authors on each paragraph start by giving a large bulk of information that is not required. They should try to restrict these introducing sentences to the minimum required.

2) Fig 5 D. I am not sure about the nomenclature here. The second panel is a clear bipolar clustered figure (not a pseudo bipolar), the third one I would called it a pseudo-bipolar spindle. The extra pole is within the main spindle.

1st Revision - authors' response

11 September 2014

Point-by-point response to the reviewer's comments

We thank the three reviewers of our original manuscript for their critical reading and for their constructive and useful comments, which we addressed in full, as detailed below. Accordingly, we also altered the manuscript in a substantial manner.

Reviewer #1

In this manuscript, Shiratsuchi and colleagues employ mass spectrometry-based proteomic analyses to identify candidate interactors of HsSAS6 and STIL. They conduct small-scale RNAi screening to address their functions in centriole formation in human cells, and identify RBM14, as a putative STIL-interacting protein, and a novel suppressor of de novo assembly of centriole protein complexes. RBM14 can inhibit centriole over-duplication in S-phase arrested U2OS cells, and its depletion induces ectopic centriole protein complexes through interactions with the STIL/CPAP complex. The ectopic centriole protein complexes form in the cytoplasm in a STIL/CPAP-dependent manner and are independent of HsSAS6. They observe that about half the complexes have the ability to nucleate microtubules during mitosis, which leads to pseudo-bipolar spindles and multipolar spindles, and defective chromosome segregation. The authors also conducted RBM14 depletion in mouse embryos and observed the same ectopic centriole protein complex formation. Overall this is an interesting manuscript with a huge amount of work. The results are consistent with the possibility

that de novo formation of MTOC can occur independently of cartwheel formation. The biggest weakness of this manuscript is that it tries to accomplish a lot, perhaps too much, and I found it extremely disjointed at times. For example, it begins with a mass spec story on procentriole formation, transitions through de novo centriole assembly, then PCM assembly, mitotic spindle formation and finishes in mouse embryos. Yet it only very partly elucidates how RBM14 actually regulates these processes mechanistically, which to me was the most salient issue. Having said this, if the manuscript is refocused adequately and addresses the following issues, it may become suitable for publication in EMBO.

> We thank the reviewer for the critical and constructive comment on our overall description in the manuscript. Prompted by this reviewer's comment, we modified the manuscript to refocus on how mechanistically RBM14 regulates centriole/centrosome biogenesis, addressing the comments raised by this and other reviewers on this issue (please see below). In parallel, we decided to remove description on the in vivo mouse work from the result section, but only briefly mentioned it in the discussion (page 21). In addition, we largely cut off the work on multipolar spindle and chromosome segregation from the main figures and text, but instead reorganized the figures (Figures 6–8 in the original manuscript) into one figure (Figure 6 in the revised manuscript) so as to focus on formation of procentriole-like structures induced by RBM14 depletion. We therefore believe that the revised manuscript now become more succinct focusing on the role of RBM14 in centriole/centrosome biogenesis.

We also restricted the description to minimum by reducing some extra explanation throughout the manuscript to make our main claims more explicit and effective.

Major issues:

1) The authors need to clarify if endogenous RBM14 localizes to centriole or PCM foci to any reasonable extent. The authors should provide quantitative data using antibodies targeting the endogenous protein and show that the observed labeling goes away after RBM14 RNAi treatment.

> We agree with the reviewer that it is important to quantify the fraction of endogenous RBM14 localizing to centrioles and the efficiency of RBM14 RNAi. As mentioned in the original manuscript, we confirmed the specificity of the antibodies against RBM14 by using siRNA targeting RBM14 (Fig.E1A, and Fig. E2C (new E2E)). We found that expression levels of endogenous RBM14 were significantly reduced up to ~15.4% of that in control cells (new data, Fig.E1A, page 55). We also found that endogenous RBM14 proteins rarely localize to centrioles marked by centrin antibodies (1.1±1.1% and 1.1±1.1% of interphase and mitotic U2OS cells, respectively, n=90, upper panels in new Fig. E2E). This is in line with the observation that RBM14 proteins act in the cytoplasm rather than at centrioles to suppress ectopic formation of centriolar protein complexes. This new piece of data is reported in the revised manuscript (page 10 and 55, Figs. E1A and E2E).

2) The authors identified RBM14 by mass spectrometry as a putative interactor of STIL and showed it had a function in centriole duplication. It is also mentioned that HsSAS-6 was also analyzed in the same way and that putative interacting proteins were tested by RNAi. I think this data should be included in the manuscript.

> We agree with this reviewer's suggestion that it would be more informative to indicate HsSAS-6-interacting proteins identified by our mass spectrometry (MS) analyses. We accordingly added the description in the list of our MS analyses (new data, Table E1). We show the list of 10 proteins that were identified as high-rank candidates for HsSAS-6-interacting proteins in our MS experiments. Please note that we addressed their function for centriole formation in human U2OS and HeLa cells by targeting each protein with 3 different siRNAs and found that RBM14 depletion was the only case to show a defect in centriole biogenesis. This result is now mentioned in the legend of Table E1 (page 55).

3) The authors mention a lot of "data not shown" suggesting that the transcriptional activity of RBM14 is not needed for its role in centrosome assembly. I am not sure I totally agree with this argument. In fact, the localization of RBM14 suggests that its activity may lie in the nucleus and the deletion of its RNA-binding domain prevents its ability to rescue the loss of endogenous RBM14. Is there a surgical mutation than can insert in RBM14 that would allow them to further rule-out an

indirect transcriptional role? Alternatively, the authors could further substantiate their biochemical data on the RBM14/STIL interaction and the effect of STIL on the STIL/CPAP complex assembly.

> We thank the reviewer for pointing this out. We mentioned a “data not shown” (page 7) for the experiment using cycloheximide to examine the effect of translation inhibition on the ectopic formation of centriolar protein complexes in RBM14-depleted cells. We now indicated the corresponding data showing that RBM14 depletion leads to excess formation of centrin foci even in the presence of cycloheximide (new data, Fig. E1G, page 7 and 56–57). Furthermore, given that RBM14[C] lacking the RNA-recognition motif (RRM), but binding to STIL, retained the ability to rescue the loss of endogenous RBM14 (Fig. 2A), it is likely that RBM14 functions in centriole biogenesis independently of its indirect transcriptional role. In line with this, we also indicated that expression of RBM14-NES which localized only in the cytoplasm efficiently suppressed HU-induced amplification of centrin foci (Fig. 2B and D). Furthermore, we added new data establishing that expression of RBM14-NES rescued the phenotype provoked by depletion of endogenous RBM14 (new data, Fig. E2G, page 10–11 and 60), suggesting that cytoplasmic localization of RBM14 is crucial for its function to suppress amplification of ectopic centriolar protein complexes.

> As suggested by this reviewer, we also conducted in vitro pull-down assay to test whether STIL-interacting proteins, RBM14 [C] and CPAP [SBD], compete with each other for binding to STIL[N]. We found this to be the case indeed, further supporting the current model that RBM14 prevents the formation of STIL/CPAP complex (new data, Fig. 3E, page 12 and 47).

4) Do the authors know if CPAP/STIL and RBM14 are present in the same protein complex? Are their interactions dependent on other proteins are CPAP, STIL and RBM14 able to assemble independently of other proteins?

> This reviewer raised an important issue, which was also mentioned by the reviewer 2#. First, as mentioned above, we established that RBM14 and CPAP compete with each other for interacting with STIL at least in vitro probably because they share the overlapping region within STIL for the interaction (new data, Fig. 3E). To confirm this in vivo, we performed western blot analysis using the same STIL-IP fraction as in Fig. 3A. Although we found endogenous RBM14 proteins in the fraction again, we could not detect endogenous CPAP proteins there (new panel for CPAP in Fig. 3A). Furthermore, we repeated mass spectrometry analysis with IP fractions with STIL antibodies from 293T cells in different conditions. We reproducibly found a number of RBM14 peptides in the fractions, but failed to detect CPAP peptides in any condition (now mentioned in the figure legend of table E1, page 55). In our hands, it was impossible to detect robust interaction between endogenous STIL and CPAP out of the cytoplasmic lysate (Fig. 3A). We could detect the STIL/CPAP complex formation only when either of them was overexpressed in human cells. Together, we propose that STIL forms a complex with RBM14 in the cytoplasm rather than with CPAP, and that the STIL/CPAP complex formation may be tightly regulated for procentriole formation. This result is now mentioned in the result section (page 11–13).

5) The authors draw the conclusion that cytoplasmic RBM14 could efficiently suppress centriole amplification in S-phase arrested U2OS cells. I feel the foundation upon which this conclusion is based is somewhat weak. They overexpress full-length RBM14 and the C-terminal region of RBM14 (which was suggested to localize to the centrosome, albeit not very efficiently) and found they both suppressed HU-induced centriole amplification. On the other hand, RBM14-PACT, which localizes to the centrosome, did not suppress HU-induced centriole amplification as efficiently as RBM14-NES. From this reviewer's perspective, the two observations seem to contradict each other, and since most of the RBM14-PACT localizes to the nucleus, it is possible that the amount of RBM14-PACT at the centrosome and in the cytoplasm is less than the RBM14-NES, and that's why RBM14-PACT is not as efficient at suppressing centriole over-duplication. Since RBM14 [C] localizes to the centrosome and it is as efficient as RBM14 [FL], I'm just not convinced by their conclusion.

> This reviewer is correct in raising the possibility that expression levels of RBM14-PACT might be less than those of RBM14-NES. According to this suggestion, we quantified and compared the expression levels of RBM14-NES and RBM14-PACT proteins in the cytoplasm. Using western blot analysis, we found the amount of RBM14-NES expressed in the cytoplasm more than that of RBM14-PACT (~1.4 fold, newly mentioned in the figure legend of Fig. 2D, page 45–46). It is therefore possible that the difference in the ability to suppress HU-induced centriole amplification

between RBM14-NES and RBM14-PACT results from the difference in their cytoplasmic expression levels. Based on the observation, we corrected the sentence mentioning the ability of RBM14-PACT for suppression of HU-induced centriole amplification compared with that of RBM14-NES (line 21 of page 10 in the revised manuscript). However, given that expression of RBM14-PACT which efficiently localized to centrioles did not increase the extent of the suppression for centriole amplification and also that expression of RBM14-NES which localized to the cytoplasm, but seemingly not to centrioles, suppressed the centriole amplification as is the case for expression of the native full-length, we remain of the opinion that RBM14 likely acts on centriole biogenesis in the cytoplasm rather than at centrioles or in the nucleus. Consistently, RBM14[C] which more frequently localizes to the centrosome suppressed HU-induced centriole amplification only as efficiently as RBM14 full-length did.

6) Normal procentrioles are generated in the process of centriole over-duplication in the S-phase arrested U2OS cell assays, which apparently are different from the ectopic centriole protein complexes generated after RBM14 depletion. In this case the authors should address the mechanistic link between normal procentriole formation and ectopic centriole protein complex formation affected by RBM14.

> We thank this reviewer for raising this issue. First, we demonstrated that both STIL and CPAP are required for centriole amplification in S-phase arrested cells in Fig. E5B (Fig. E4E in the original manuscript) as well as ectopic formation of centriolar protein complexes induced by RBM14 in Fig. 3G (Fig. 3F in the original manuscript). Second, in addition to Fig. 3F (Fig. 3E in the original manuscript), using in vitro competition assay, we now show direct evidence that binding of RBM14 to STIL competes with the complex formation of STIL and CPAP (new data, Fig. 3E, page 12). Furthermore, we found that the STIL/CPAP complex formation in the cytoplasm was enhanced in the S-phase arrested U2OS cells upon treatment with HU (new data, Fig. E4C, page 63). Based on these observations, we assume that RBM14 could inhibit centriole amplification in the S-phase arrested cells by suppressing formation of the STIL/CPAP complex in the cytoplasm. We therefore propose that formation of the STIL/CPAP complex would be critical for both centriole amplification and ectopic formation of centriolar protein complexes and thus be the mechanistic link that is targeted by RBM14 in these two processes. However, the former requires HsSAS-6 to form normal procentrioles in S-phase arrested cells (Fig. E5B) whereas the latter does not (Fig. 4A). This likely reflects the difference in structure between normal procentrioles and amorphous centriolar protein complexes induced by RBM14 depletion. This point is now discussed in the revised manuscript (page 21–22). To further address the mechanistic link between the two processes focusing on the function of RBM14 would be indeed of interest in the future, but arguably beyond the scope of this study as the editor mentioned.

7) The authors should test whether RBM14 overexpression will affect centriole duplication in cycling cells. Also, the authors found that over-expression of RBM14 did not affect formation of multiple procentrioles induced by PLK4, STIL, HsSAS6 overexpression. The difference may be caused by different mechanisms behind centriole overduplication caused by S-phase arrest or PLK4/STIL/SAS6 over-expression in U2OS cells, and it would be good to know where RBM14 is functioning in the process.

> We reported in the original manuscript that overexpression of RBM14 had only minor effect on canonical centriole duplication in cycling cells (Fig. E2B). We therefore concluded that RBM14 would not play a critical role in canonical procentriole formation or formation of multiple procentrioles induced by Plk4, STIL, HsSAS-6 overexpression. This is in line with the fact that molecular requirements for the concurrent formation of multiple procentrioles seems to be similar to those for canonical procentriole formation (Kleylein-Sohn J. et al. (2007)). On the other hand, it has been recently suggested that there are different mechanisms in centriole amplification in S-phase arrested cells from the canonical procentriole formation pathway (Kuriyama et al. (2007), Prosser SL. et al. (2009), Tsang WY. et al. (2009)). Our data suggest that RBM14 overexpression suppresses centriole amplification in S-phase arrested cells presumably by acting on ectopic formation of the STIL/CPAP complex in the cytoplasm. Indeed, we found that the STIL/CPAP complex formation in the cytoplasm was much enhanced in the S-phase arrested 293T cells upon treatment with HU compared with in cycling cells (new data, Fig. E4C, page 63). We assume that formation of the STIL/CPAP complex is strictly regulated in the cytoplasm in the process of canonical procentriole formation and that is why RBM14 overexpression did not significantly affect canonical centriole

duplication in cycling cells. This point is also mentioned in the revised manuscript (page 21–22).

8) *From my perspective the work on multipolar spindle, chromosome segregation and in vivo mouse is not necessary and does not really increase the impact of the manuscript. I would consider removing it and to focus on the role of RBM14 in centriole/PCM assembly.*

> We thank this reviewer for the constructive suggestion. As mentioned above, we modified the manuscript to refocus on the role of RBM14 in centriole/centrosome biogenesis, addressing the comments raised by this and other reviewers. In parallel, we decided to remove description on the in vivo mouse work from the result section, but only briefly mentioned it in the discussion (page 21). In addition, we largely cut off the work on multipolar spindle and chromosome segregation from the main figures and text, but instead reorganized the figures (Figures 6–8 in the original manuscript) into one figure (Figure 6 in the revised manuscript) so as to focus on formation of procentriole-like structures induced by RBM14 depletion. We therefore believe that the revised manuscript now becomes more succinct focusing on the role of RBM14 in centriole/centrosome biogenesis.

9) *The authors should clarify if the electron dense structures they observe in absence of RBM14 require PCM components for their assembly.*

> We thank the reviewer for this valuable suggestion. To examine the dependency of PCM for ectopic formation of centriolar protein complexes induced by RBM14 depletion, we compromised PCM assembly by treatment with siRNA targeting Cep192 on top of RBM14 depletion in U2OS cells. We found that Cep192 depletion did not significantly affect centriole amplification in RBM14-depleted cells whereas it efficiently suppressed assembly of PCM proteins such as g-tubulin (new data, Fig. E5D, page 14 and 64). We thus conclude that assembly of PCM components is not critical for this process.

10) *The authors argue that the amorphous structures they observe when depleting RBM14 are somewhat organized like real centrioles (based on distance measurements), yet there are no centrioles. Most problematic here is that they use conventional microscopy to make these claims. I am not sure what point they want to make are the structures they see organized or amorphous or both?*

> We thank the reviewer for the comment. As this reviewer pointed out, the data in Fig.6D showing the placement of centriolar markers in the ectopic centriolar protein complexes induced by RBM14 depletion would not be sufficient to judge the structural feature. However, in figures 7 and 8 in the original manuscript, using long-term live cell imaging and CLEM (Correlative Light-Electron Microscopy) analyses, we provided evidence that, upon RBM14 depletion, modest population of ectopic centriolar protein complexes incorporates HsSAS-6 in the cytoplasm and some of them, at least, become procentriole-like structures. As shown in Fig. 8B, E6E and F (new Fig.6H, Fig.E8A and B), CLEM analysis revealed that ectopic GFP-centrin aggregates that assemble a mitotic spindle in RBM14-depleted cells appeared to be a centriole-like structure in a cylindrical arrangement. We also provided another example in Fig. E7 (new Fig. E8C), further confirming this claim. Since formation of an extra spindle pole in RBM14-depleted cells required ectopic HsSAS-6 (Fig. 8D and F (new Fig. 6A and C)), we postulate that centriole-like structures that we detected in the CLEM analysis likely reflect ectopic HsSAS-6-containing procentriole-like structures observed in immunofluorescence analysis. In addition, given that HsSAS-6-positive centriolar protein complexes contained most centriolar markers and presented almost appropriate placement of centriolar markers (new Fig. 6D), we assume that the HsSAS-6-containing centriolar protein complexes in RBM14 depleted cells are likely organized procentriole-like structures whereas the other centriolar protein complexes without HsSAS-6 are just amorphous structures. Overall, these results support the notion that RBM14 depletion induces formation of ectopic centriolar protein complexes (amorphous structures), some of which can assemble into procentriole-like structures presumably by incorporating HsSAS-6 and function as spindle poles. As mentioned above, to make this claim more explicit, we simplify the description and combine these data into one figure (Fig. 6, page 16–20).

Minor issues:

1) *The authors conclude that ectopic centriole protein complex forms at S-phase, because Lovastatin-induced G1-phase arrest inhibited amplification whereas HU-induced S-phase arrest did not. The conclusion can also be supported by observe complex formation of cycling cells accompanied by PCNA staining marking S-phase to rule out artificial affect caused by cell-cycle arrest.*

> We agree with this reviewer that it would be more convincing to conduct PCNA staining on RBM14-depleted cells to make sure that ectopic centriolar protein complexes form in S-phase. To understand the cell cycle phase where formation of ectopic centriolar protein complexes begins, we looked at the phenotype induced by RBM14 depletion at earlier time point, 24 hours after RNAi treatment in U2OS cells. We found that there was a clear tendency that centriole amplification was more frequently found in PCNA-positive S-phase cells than in PCNA-negative G1-phase cells (36.7±3.3% and 14.4±4.0%, respectively, n=90, new data, Fig. E5G, page 14, 65), supporting the claim that ectopic centriolar protein complexes form in S-phase.

2) *The authors suggested that 54% of the centriole protein complexes appeared to harbor microtubule nucleation activity in mitosis. The experiment seems to be done only once and it needs repeating, because it doesn't fit with the observation that only 40% of centriole protein complexes contain γ -Tubulin. I'm curious about how the remaining 14% of complexes are able to nucleate microtubules.*

> Prompted by the comment from this reviewer, we repeated the experiment two more times in Fig. 5 addressing to what extent the centriolar protein complexes have microtubule nucleation activity in mitosis. The quantification out of three independent experiments reveals that 46.0±5.3% of them appears to have microtubule nucleation activity in mitosis (new data, mentioned in the revised manuscript (page 16). This value is comparable to the population of the centriolar protein complexes containing g-tubulin (39.3±2.4%). We speculate that the remaining ~7% of the complexes might also have very weak signal of g-tubulin or at the levels under detection limit by immunofluorescence and thus might be able to marginally nucleate microtubules.

3) *The authors observed that some small Centrin foci fused with each other and grew to the size comparable with pre-existing centrioles. Is size important for these ectopic complexes to form spindle poles? And how are the foci moving to each other? Is it microtubule dependent? In movie E1/2/3, it seems to me that ectopic centriole protein complexes have a lot of interactions with pre-existing centrioles. It may suggest that centrosomal STIL and CPAP are also responsible for ectopic centriole protein formation.*

> This reviewer suggested interesting possibilities on the dynamics of ectopic centriolar protein complexes in RBM14-depleted cells. First, we could not find robust correlation between the size of ectopic centriolar protein complexes and their ability to nucleate microtubules although too small foci did not form a spindle pole during mitosis. For example, in Fig. 8B (new Fig. 6H), distinct centrin foci, but relatively smaller than those of preexisting centrioles, could still organize a spindle. We therefore assume that whether or not the structures incorporate HsSAS-6 matters for the microtubule nucleating activity to form spindles. Second, according to the suggestion from this reviewer, we tested the significance of the microtubule network on the behavior of ectopic centriolar protein complexes by using nocodazole treatment. Interestingly, we found that microtubule depolymerization by nocodazole treatment did not significantly affect ectopic formation of centriolar proteins complexes in RBM14-depleted cells (new data, Fig. E5F, now mentioned in page 14 of the revised manuscript), suggesting that the microtubule network itself is not crucial for this process. Using live-cell imaging, we confirm that our nocodazole treatment well decreased back-and-forth movement between the centrosome and cytoplasm of ectopic centriolar protein complexes. This is consistent with the fact that microtubules facilitate centrin foci movement shuttling at the vicinity of centrosomes (Rafelski, S.M. et al (2011)). Based on these observations, we assume that the interaction of the ectopic centriolar protein complexes with preexisting centrioles is not necessary for its assembly.

4) *Does RBM14 interact with other centriole duplication and PCM proteins? The authors could use mass spectrometry-based proteomic analysis to identify candidate interactors of RBM14. Alternatively, the authors could confirm the interaction between RBM14/STIL using reciprocal Co-IP experiments and blotting for additional centriole/PCM proteins?*

> We thank this reviewer for the suggestion. We sought to investigate whether RBM14 interacts with other centriole/PCM components. First, RBM14 antibodies were tested for immunoprecipitation (IP) to bring down endogenous RBM14 and its interacting proteins, but unfortunately they did not work well for IP. Therefore, we instead conducted Co-IP experiments using 293T cells expressing RBM14-Flag full-length and C-terminal fragment, both of which were shown to interact with HA-STIL (Fig. E3). We efficiently detected endogenous STIL proteins in the IPed fraction of RBM14[C]-Flag which was expressed much more than the full-length. Interestingly, among some centriole/PCM proteins that we tested, we found reasonable amount of g-tubulin in the fraction (new data, Fig. E5E, page 14 and 65). Given that PCM components including Cep192 and g-tubulin are not required for ectopic formation of centriolar protein complexes in RBM14-depleted cells (new data, Fig. E5D, page 14 and 64) and also that RBM14 overexpression did not affect PCM assembly (new data, Fig. E2C, page 9 and 59), we are still not sure the biological significance of the interaction of RBM14 with g-tubulin. We believe that this should be an important future line of research. This point is now described in the revised manuscript (page 14).

5) *The authors repeatedly use words like "interestingly", "strikingly", and "surprisingly" which weighs heavily on the reader... I would try to use them more sporadically and let the readership qualify the observations.*

> We agree this reviewer that it would be better not using “emphatic words” too many times. We thus restrict such words and use them only where appropriate throughout the manuscript.

6) *The authors mention several times in the manuscript that they will analyze the "molecular architecture". I think this is difficult to do using the methodologies used in the manuscript and the statement needs to be toned down.*

> We apologize for not having been sufficiently accurate in the wording of our initial submission. We altered the wording in the revised manuscript, replacing the term “molecular architecture” – which was incorrect- by the term “molecular components” –which in our view faithfully reflects the analysis that we mean to perform (page 16, 17).

7) *The authors claim that RBM14 prevents de novo centriole assembly. With the data shown I think this is an overstatement and the title should be changed.*

> The title in the original manuscript was “RBM14 prevents de novo assembly of centriolar protein complexes and maintains mitotic spindle integrity” which does not necessarily mean that RBM14 prevents de novo centriole assembly. Nevertheless, as this reviewer pointed out, if the title sounded like that RBM14 depletion always induces de novo assembly of complete centrioles, it might be an overstatement. We therefore decide not to use “de novo” in the title and abstract. We believe that the current title “RBM14 prevents assembly of centriolar protein complexes and maintains mitotic spindle integrity” reflects what we would like to claim out of the data shown in the revised manuscript.

Referee #2:

Centrosomes consist of a pair of centrioles surrounded by the pericentriolar material (PCM) that nucleates and anchors microtubules. Centrioles recruit and organize the PCM. Centriole duplication involves the growth of a new centriole next to a pre-existing centriole and it occurs only once per cell cycle in most proliferating cells. However, in some specialized cells (e.g. multiciliated cells), hundreds of centrioles form almost simultaneously via an acentriolar (de novo) pathway. In this manuscript, Shiratsuchi et al. attempt to persuade the readers that RBM14 acts as a novel suppressor that prevents de novo assembly of "centriolar protein complexes" in proliferating cell cytoplasm. The cytosolic "centriolar protein complex" is a loose term, which has not yet been precisely defined and acknowledged by the Centrosome society. Currently, the composition/structure/function of this complex and its relation to the process of centriole duplication are not clear. Using the mass spectrometry-based proteomic analysis, the authors first identified that RBM14 is a STIL-interacting protein. Interestingly, depletion of RBM14 can induce the formation of ectopic electron-dense

amorphous structures in the cytoplasm that possibly contain the "centriolar protein complexes". Further studies showed that the formation of such structures seems not require hSAS-6-containing cartwheel and these structures can function as microtubule organization centers (MTOCs). Intriguingly, a portion of these ectopic "centriolar protein complexes" can also assemble procentriole-like structures containing hSAS-6 in RBM14-depleted cells. Furthermore, they also showed that the interaction of RBM14 with STIL seems to prevent the formation of the STIL/CPAP complex in the cytosol. This would be an interesting finding if confirmed that will provide novel and significant insight into the composition and function of this poorly defined complex. The quality of the data is high and the study nicely combines biochemical work and functional analyses. However, some conclusions lack sufficient experimental support, which needs to be further addressed. The following concerns should be addressed prior publication.

> We thank this reviewer for supporting this manuscript and constructive comments/suggestions towards publication.

Major points:

1) The authors observed that depletion of RBM14 can induce ectopic formation of centriolar protein complexes (Fig. 1). It is also possible that these centriolar protein complexes could be produced from the fragmentation of preexisting centrosomes in siRBM14-treated cells. How do the authors distinguish between these two possibilities?

> This reviewer raised an important issue. In our view, a few lines of experiments that we demonstrated in this study can distinguish these two possibilities. First, we did not detect any significant reduction in the expression levels of centriole/centrosome markers at the preexisting centrosomes in RBM14-depleted cells, compared to those in control cells, whereas ectopic formation of centriolar protein complexes substantially occurred in the cytoplasm (Fig. 1). In addition, we did not observe any sign of fragmentation of preexisting centrioles/centrosomes in the staining pattern of centriole/centrosome markers. Second, the live-cell imaging with cells expressing fluorescence-labeled centrin or HsSAS-6, described in Figs. 4 and 7 (new Figs. 4 and 6), showed that ectopic GFP-centrin or HsSAS-6 foci were efficiently formed in the cytoplasm, fusing with each other even far from preexisting centrioles, when the signal intensities of those at preexisting centrioles were not altered in RBM14-depleted cells. Third, as mentioned above, when nocodazole treatment with RBM14-depleted cells disrupted any interaction between ectopic centriolar protein complexes and preexisting centrosomes, we still found that ectopic centriolar protein complexes grew in the cytoplasm (new data, Fig. E5F, page 14, 65). Overall, these lines of experiments allow us to conclude that formation of ectopic centriolar protein complexes happens in RBM14-depleted cells unlikely due to fragmentation of preexisting centrosomes. This point is now mentioned in the revised manuscript (page 14).

2) To further support the concept that the electron-dense amorphous structures are the sites that contain the centriolar protein complexes in RBM14-depleted cells, the authors need to provide either the CLEM (correlative Light-Electron Microscopy) or immunogold-EM images with antibodies against CPAP and STIL.

> We thank this reviewer for the valuable suggestion. Prompted by the suggestion, we conducted CLEM analysis using U2OS cells expressing GFP-CPAP. Similar to what we observed in Fig.1C, RBM14 depletion significantly induced ectopic formation of GFP-CPAP foci in the cytoplasm (new data, Fig. 4F, page 15 and 50). In such condition, we performed CLEM analysis and found that most of the ectopic GFP-CPAP foci that reflect assembly of centriolar protein complexes appeared to be electron-dense amorphous structures (new data, Fig. 4G, page 15 and 50). These data further support the notion that the amorphous structures are the sites that contain the centriolar protein complexes in RBM14-depleted cells. Although we also sought to examine the amorphous structures in RBM14-depleted cells using immunogold-EM with antibodies against CPAP and STIL, in our hands, these antibodies did not work well for this experiment. These new findings are now reported in the revised manuscript (page 15).

3) Overexpression of full length RBM14 or RBM14[C] had very minor effect on centriole duplication, while it did dramatically affect centriole amplification in hydroxyurea-treated cells arrested in S phase (Fig. 2). These findings lead the authors to propose that RBM14 may suppress centriole amplification in prolonged S-phase cells. However, endogenous RBM14 (RNA-binding

motif protein 14) is mainly detected in the interphase nucleus but weakly in the cytosol by IF analysis (Fig. E2C). To clearly demonstrate that the cytosolic RBM14 could efficiently inhibit centriole duplication in the S-phase cells, the authors should perform Western blot analysis on a sample prepared from both nuclear and cytosolic fractions (not a total lysate) and show that a significant amount of endogenous RBM14 protein does exist in the cytosol.

> We agree with this reviewer that the amount of cytosolic fraction of endogenous RBM14 should be addressed given its significance in inhibiting centriole amplification in S-phase arrested cells. We extracted nuclear and cytosolic fractions separately from U2OS cells and analyzed them by Western blot analysis. As shown in Fig. E2F (new data, page 10 and 60), the separation of cytosolic fraction from the nuclei was successfully done by monitoring the amount of Histone in those fractions. In the cytosolic fraction, we detected a substantial amount of endogenous RBM14 (approximately ~55% of total RBM14 proteins), which is compatible with the notion that cytoplasmic RBM14 acts on the excess STIL/CPAP complex and inhibits centriole amplification in the S-phase arrested cells. This new piece of data is now mentioned in the revised manuscript (page 10). Furthermore, we added new data establishing that expression of RBM14-NES rescued the phenotype provoked by depletion of endogenous RBM-14 (Fig. E2G, page 10–11, 60), confirming that cytoplasmic localization of RBM14 is crucial for its function to suppress amplification of ectopic centriolar protein complexes.

4) CPAP was previously reported to be a STIL-interacting protein. Interestingly, the CPAP-interacting domain in STIL is overlapped with the region binding to RBM14. The authors thus propose that the direct binding of RBM14 and STIL may perturb the function of STIL in centriole duplication possibly via disruption of the CPAP-STIL complex. However, the data presented in Fig. 3E are not strongly enough to support this conclusion. The authors should provide competing IP experiments by transfecting various amount of Flag-RBM14-FL with fixed amount of GFP-CPAP into cells and analyze the ratio of CPAP and STIL with increasing amount of RBM14. Furthermore, the authors should redo the co-IP experiment in Fig. 3A using the lysates prepared from the cytosolic fraction instead of total cell lysates and examine endogenous RBM14, STIL, and CPAP in the complex with at least two different antibodies (STIL and RBM14) for IP.

> We thank this reviewer for the valuable suggestion. To gain more direct evidence, we established in vitro competing assay where we added increasing amount of purified CPAP fragment containing STIL-interacting domain against the fixed amount of STIL[N]/GST-RBM14[C] complex. Importantly, we found that the CPAP fragment and GST-RBM14[C] competed with each other for binding to STIL[N] (new data, Fig. 3E, page 12 and 47). This result suggests that direct binding of RBM14 to STIL competes with the complex formation of STIL and CPAP. This new finding is now described in the revised manuscript (page 12). Although we intended to perform competing experiments in vivo as suggested by this reviewer, it was technically unfeasible to express various amount of RBM14 like in a linear fashion in cells by transfecting increasing amount of RBM14-Flag or using mammalian expression vectors with differently modified CMV promoters.

> We apologize for not having explained more appropriately about the method for the IP experiments performed in this study. As written in the method section of the original manuscript, for the IP experiments, we used a lysis buffer (20 mM Tris/HCl pH7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% NP40 and 1/1000 protease inhibitor cocktail) to prepare soluble fraction which contains the cytosolic fraction, but not the nuclear fraction. This was confirmed by western blot analysis using antibodies against Histone (new data, Fig. E2F, page 10 and 60). We thus altered the wording in the revised manuscript, replacing the term “total lysate” –which was confusing- by the term “input (soluble fraction)” for the IP experiments. Therefore, the IP experiment described in Fig. 3A was done with the lysates prepared from the cytosolic fraction. We performed western blot analysis using the same IP fraction with STIL antibodies. Although we found endogenous RBM14 proteins in that fraction again, we could not detect endogenous CPAP proteins there (a new panel for CPAP in Fig. 3A, page 11). Furthermore, we repeated mass spectrometry analysis with IP fractions with STIL antibodies from 293T cells in different conditions. We reproducibly found a number of RBM14 peptides in the fraction, but failed to detect CPAP peptides in any condition (now mentioned in the figure legend of table E1, page 55). In our hands, it was impossible to detect robust interaction between endogenous STIL and CPAP out of the cytosolic lysate (Fig. 3A). We could detect the STIL/CPAP complex formation only when either of them was overexpressed in human cells. On the other hand, we also tested the reciprocal Co-IP using RBM14 antibodies to bring down endogenous RBM14 and its interacting proteins, but unfortunately it did not work well for IP. Therefore, we instead conducted Co-IP experiments using 293T cells expressing RBM14[C]-Flag

which was shown to interact with HA-STIL (Fig. E3) and expressed much more than the full-length. We detected endogenous STIL proteins, but not CPAP proteins, in the IPed fraction. (new data, Fig. E5E, page 14 and 65). Together, we propose that STIL forms a complex with RBM14 in the cytoplasm rather than with CPAP, and that the STIL/CPAP complex formation may be tightly regulated so as to happen only at the assembly site of procentrioles. This issue is now discussed in the revised manuscript (page 21–22).

5) If the binding of RBM14 to STIL could disrupt the cytosolic CPAP-STIL complex as the authors' proposed, it is reasonable to speculate that ectopically expressed RBM14-FL may also sequester endogenous STIL from the CPAP-STIL complex which resulted in inhibiting centriole duplication. However, it appears not to be the case (Fig. E2B, E4C), why? Furthermore, the authors proposed that the ectopic centriolar protein complexes induced by RBM14 depletion could be the sites for PCM recruitment (Fig. 1E, Fig.E1J) and microtubule nucleation (Fig. 5B). What will happen if RBM14 is overexpressed? Does overexpression of RBM14 inhibit PCM recruitment and MTOC activity?

> Based on our results, formation of the STIL/CPAP complex seemed to be very limited in the cytoplasm in the process of normal centriole duplication, while the STIL/RBM14 complex was well detected in the cytoplasmic fraction (new data, Fig. 3A, Table E1, page 46, 55). In this case, it is therefore possible that formation of the STIL/CPAP complex mainly occurs just at the assembly site of procentrioles. On the other hand, since the complex formation was highly enhanced when cells were arrested in S-phase by HU treatment (new data, Fig. E4C, page 63), we assume that, in such situation, there is substantial amount of the cytoplasmic STIL/CPAP complex that can be targeted by cytoplasmic RBM14 whereas that is not the case in normally cycling cells. We thus speculate that this could be a plausible reason why RBM14 overexpression affected centriole amplification during S-phase arrest, but not canonical centriole duplication (Fig. E2B) and formation of centriolar rosettes (Fig. E4C (new Fig.E2D)). This point is now discussed in the revised manuscript (page 21–22).

> Prompted by the suggestion from this reviewer, we addressed the influence of RBM14 overexpression on PCM recruitment and MTOC activity. We found that RBM14 overexpression did not affect PCM assembly and microtubule nucleation during mitosis (new data, Fig. E2C, described in page 9 and 59), suggesting that RBM14 does not directly regulate these processes.

Minor points:

1) Why do the percentages of centrin foci (>4) differ between Fig. 1A (~33%) and Fig. 2A (~18%)? Different siRNA treatments? Please clarify it.

> The siRNA used in Fig.2A targets the 3'UTR of RBM14 to deplete only endogenous RBM14 and assess the function of transfected exogenous deletion mutants of RBM14. For the rest of RNAi experiments including Fig. 1A, siRNA against the ORF of RBM14 was used, which depleted endogenous RBM14 more efficiently and thus induced amplification of centrin foci more frequently than the siRNA targeting the 3'UTR. This is explained more explicitly in the method section and figure legend of Fig. 2A of the revised manuscript (page 26, 44–45).

2) The authors need to provide statistic SEM in Fig. 7 and Fig. 8

> We thank the reviewer for pointing this out. We provided SEM where necessary in Fig. 7 and 8 (new Figs .6A, E6C, E7A) of the revised manuscript. However, for some experiments counting cells out of live-cell imaging, we judged that it is not appropriate to provide SEM due to limitation of N number (new Figs. 6F, E6A, E7C).

3) The Figure 9 legend (hypothetic model) is not clear. The authors need to provide some explanation in the figure legend that links their experiments to when, where and how RBM14 functions on the centriolar protein complexes.

> We thank the reviewer for the suggestion. We added some explanation in the figure legend to show our claims for the RBM14 function on formation of the centriolar protein complexes in a clearer fashion (new Fig.7, page 54).

Referee #3:

The work of Shiratsuchi and colleagues, entitled "RBM14 prevents de novo assembly of centriolar protein complexes and maintains mitotic spindle integrity" describes the identification of a novel protein, RBM14, that inhibits the de novo formation of centriole like structures.

The authors identified RBM14 as a STIL interacting protein, which is a well characterised centriole duplication protein. RNAi approaches showed that in U2OS RBM14 depleted cells, extra centrosome like structures (centrin, acetylated tub positive structures) were formed. They convincingly ruled out a role of RBM14 in translation that would explain the appearance of these structures. Further work showed that RBM14 prevents the formation of STIL-CPAP complexes and the authors have mapped the domain of interaction. Surprisingly, they show that these centriole-like structures lack Sas-6 and even more surprisingly Plk4 activity. To gain information on the ultra structure level of these structure the authors perform EM analysis and propose that these structures lack a cartwheel and so that they form independently of the canonical centrosome duplication pathway. Nevertheless, these structures act as MTOCs, and if they incorporate Sas-6 they form centriole like structures. They behave as

extra centrosomes and in cases of spindle formation these can fail to be bipolar and to correctly segregate chromosomes. Further analysis of mouse embryos depleted of RBM14 confirms in vivo the results obtained in vitro.

This is a very nice paper that identified a novel function for a novel protein in the inhibition of de novo centriole assembly that will be of interest to the centrosome/MT and mitotic fields. Most of the data presented here is of very good quality, contains the adequate controls, quantification and interpretations. I am therefore recommending publication by the EMBO J. However, some of the observations and statements in this version, require extra clarification before publication.

> We thank this reviewer for supporting this manuscript and constructive comments/suggestions towards publication.

Major points

1) The authors convincingly show that RBM14 is involved in the inhibition of the assembly of these centriole like structures in the cytoplasm. However they claim that these structures are not dependent on the presence of Sas-6 or Plk4. I am slightly concerned by these observations. Can the authors provide compelling evidence that Plk4 and Sas-6 have been depleted to lower levels consistent with inhibition of centriole duplication. These proteins (specially Plk4) are low abundant, and so even a reduction might not be sufficient to completely abolish their functionality. So the authors should provide unequivocally evidence that the RBM14 depletion leads to the formation of centriole like structures independently of Sas-6 or Plk4. In Fig E4 they have shown that plk4 RNAi results in the reduction of Sas-6 foci, but are these conditions the same used to analyse the induction of these structures in the absence of RBM14? How about the Sas-6 depletion? Also, for how long were these cells treated for? Is it possible that they are just being arrested prior to S phase? This should be investigated. This is particularly important regarding the identification of RBM14 protein as a Sas-6 and Stil interactors (First paragraph of results section). How to explain this?

> We agree with this reviewer that it is important to provide solid evidence that RBM14 depletion leads to the formation of centriolar protein complexes independently of HsSAS-6 or Plk4. Just to make sure, we would like to mention that RBM14 was identified as an interacting protein for STIL, but not for HsSAS-6 (new Table E1, page 55). As mentioned in the method section (page 27), for double RNAi experiments in Fig. 4A, E4D and F (new Fig. 4A, E5A and E5C), cells were treated with siRNAs for 72 hours in the same condition. We monitored the extent to which HsSAS-6 was depleted by RNAi treatment in RBM14-depleted cells using western blot analysis. We found that expression levels of endogenous HsSAS-6 were significantly reduced up to ~7.1% of that in control cells (Fig. E5A). The depletion of HsSAS-6 was as efficient as that by single HsSAS-6 RNAi treatment by which we normally observe significant defect in centriole duplication. Since it was technically difficult to monitor expression levels of endogenous Plk4 by western blot analysis due to their expressions being very low, we confirmed the efficacy by monitoring the reduction of HsSAS-6 foci as shown in Fig. E4F (new Fig. E5C). We also performed PCNA staining (to mark with S-phase cells) on cells treated with HsSAS-6 RNAi or Plk4 RNAi together with RBM14 RNAi, which showed that there was comparable population of PCNA-positive cells found in such cells, compared to that in control cells (39±3%, 39±4%, 41±5% of control, HsSAS-6- and Plk4-depleted U2OS cells;

36±3%, 39±4%, 40±6% of RBM14-depleted, HsSAS-6/RBM14-depleted, and Plk4/RBM14-depleted U2OS cell, respectively. n=150 in triplicate, new data). This result indicates that double RNAi treatment unlikely arrests cells prior to S-phase. Overall, we remain confident that depletion of HsSAS-6 and Plk4 by RNAi treatment was efficiently done in the double RNAi experiments in Fig. 4A, E4D and F (new Fig. 4A, E5A and E5C), and concluded that RBM14 loss results in the formation of centriolar protein complexes independently of HsSAS-6 or Plk4.

2) *Along the same lines the authors show in Fig 4E that these cytoplasmic structures resemble amorphous electron dense material that do not contain sas-6. These figs are extremely difficult to interpret and to distinguish. Can the authors provide figures of better quality? And the quantification of the observed structures.*

Can also the authors comment of the fact that if these structures are not really bona fide centrioles with a cartwheel, how can they explain that they are able to behave as such since they recruit PCM and nucleate MTs? If these structures are formed independently of Plk4/Sas-6, they only contain STIL and CPAP as core centriole duplication factors? When is centrin incorporated, and Cp110? This should be characterised in a timely defined way.

>We thank this reviewer for the suggestions. We first replaced the EM pictures in Fig. 4E with the retaken ones illustrating the feature of microtubule structures more clearly. In addition, quantification of the diameter of amorphous structures, compared with that of preexisting centrioles is now added in the legend of Fig. 4E (new data, pre-existing centrioles: 225±8 nm (n=10), amorphous electron dense structures: 130±11 nm. (n=16), page 49–50). Moreover, CLEM analysis also confirmed that these amorphous structures represent ectopic CPAP foci (new data, Fig. 4F and G, page 15, 50).

As far as we tested, among evolutionarily conserved core centriole duplication factors, STIL and CPAP are the only proteins that are essential components for ectopic centriolar protein complexes formed in RBM14-depleted cells. In contrast, in the original manuscript, we already showed that Plk4 and HsSAS-6 are dispensable for this process. We also found that ectopic formation of centrin foci induced by RBM14 depletion did not require Cep192, g-tubulin (new data, Fig. E5D, page 14, 64) or Cep152 (21±3% of RBM14/Cep152-depleted cells harbored >4 centrin foci, compared with 6±1% of Cep152-depleted cells, n=90 in triplicate, new data).

Formation of ectopic centriolar protein complexes takes place in S-phase, which was monitored by assembly of centrin and CP110 in the cytoplasm (Fig. 4D and new data in Fig. E5G and H, page 14, 65). We therefore assume that incorporation of these components almost concomitantly happens. Given the heterogeneity of the structures some of which lack centrin or CP110 (~25% of CP110 foci and ~45% of centrin foci lack the other, respectively, n=150, new data and Fig. E1J), we speculate that there may not be a well-defined order by which these components are incorporated into the structures. This point is now mentioned in the revised manuscript (page 21).

This reviewer also raised an interesting issue how these structures can assemble PCM and microtubules without being bona fide centrioles. It has been shown that SAS-4/CPAP provides a scaffold for cytoplasmic assembly of PCM components, which implies that PCM assembly could begin in the cytoplasm before recruitment of this complex to the centrosome (Gopalakrishnan et al, 2011). In a similar way, we propose that the STIL/CPAP complex gives a scaffold for centriolar and PCM components assembly in the cytoplasm of RBM14-depleted cells and that such complexes including g-tubulin can be functional enough to nucleate microtubules during mitosis. This is now mentioned in the discussion section of the revised manuscript (page 22).

3) *I am not sure that the incorporation of Sas-6 in 8% of these structures is sufficient to state that they become more like centrioles. Here EM is necessary to show that indeed these structures are centrioles- 9 triplets of MTs arranged in a structure that contains a cartwheel.*

> We thank this reviewer for the comment. As this reviewer pointed out, the data in Fig.6 showing the incorporation of HsSAS-6 and the placement of centriolar markers in the ectopic centriolar protein complexes induced by RBM14 depletion would not be sufficient to judge whether or not they look like centrioles. However, in figures 7 and 8 in the original manuscript, using long-term live cell imaging and CLEM (Correlative Light-Electron Microscopy) analyses, we provided evidence that, upon RBM14 depletion, modest population of ectopic centriolar protein complexes incorporates HsSAS-6 in the cytoplasm and some of them, at least, become centriole-like structures. As shown in Fig. 8B, E6E and F (new Fig. 6H, E8A and B), CLEM analysis revealed that ectopic GFP-centrin aggregates that assemble a mitotic spindle in RBM14-depleted cells appeared to be a

centriole-like structure in a cylindrical arrangement. We also provided another example in Fig. E7 (new E8C), further confirming this claim. Unfortunately, since we could not observe a cartwheel with 9-fold symmetrically arranged microtubules, we could not conclude that these structures are bona fide procentrioles judging by our CLEM analyses. Accordingly, we soften this statement in the corresponding text (page 20). However, to our knowledge, because of a technical reason, it would be almost unfeasible to observe a cartwheel in a clear fashion even at preexisting centrioles of human cells by conventional EM. Since formation of an extra spindle pole in RBM14-depleted cells required ectopic HsSAS-6 (Fig. 8D and F (new Fig. 6A and C)), we believe that centriole-like structures that we detected in the CLEM analysis likely reflect ectopic HsSAS-6-containing structures. In addition, given that HsSAS-6-containing centriolar protein complexes contained most centriolar markers in an appropriate placement (Fig. 6D), we propose that the HsSAS-6-containing centriolar protein complexes in RBM14-depleted cells are organized procentriole-like structures whereas the other centriolar protein complexes without HsSAS-6 are just amorphous structures. Overall, these results support the notion that RBM14 depletion induces formation of ectopic centriolar protein complexes (amorphous structures), some of which can assemble into procentriole-like structures by incorporating HsSAS-6.

4) *A semantic question. If the structures that form in the cytoplasm are not bona fide centrioles, can the authors used this nomenclature when they describe them. Maybe centriole like?*

> We refer to the ectopic structures containing centriolar proteins and induced in RBM14-depleted cells as “centriolar protein complexes” in this article because they would not be real centriole-like in structure, but amorphous structures harboring the activity for PCM assembly and microtubule nucleation. However, to distinguish from those structures, we refer to the centriolar protein complexes which incorporate HsSAS-6 and other centriole proteins in an appropriate placement like in bona fide procentrioles, as ‘procentriole-like structures’.

5) *All these experiments were performed in U2OS cells, which are transformed cancer cells. Can the authors deplete RPE1 cells of RBM14 and show whether these structures are present? If it is not the case, it is not a problem but it will be very informative to know if only cancer cell lines can really generate such structures.*

> We thank this reviewer for the valuable suggestion. Prompted by this suggestion, we tested whether RBM14 depletion leads to ectopic formation of centriolar protein complexes in RPE1 cells. We found this indeed to be the case (>4 centrin foci found in 16.7±4.4% of RBM14-depleted cells, compared to 3.3±1.7% of control cells, new data, Fig. E1C, page 6, 55–56). Together with significant increase of such structures in RBM14-depleted NIH3T3 cells (Fig. E8C (new Fig.E9C)), we believe that this phenomenon would not be restricted in transformed cancer cells. This new piece of data is now mentioned in the revised manuscript (page 6).

Minor points

1) *the text clear needs editing. There is too much unnecessary text in the Results section. The authors discuss some of their results in this section and this should be moved to the Discussion section. Also the authors on each paragraph start by giving a large bulk of information that is not required. They should try to restrict these introducing sentences to the minimum required.*

> We thank this reviewer for the constructive comments on description in the results section. Prompted by this, we modified and restricted the description to minimum throughout the section mainly by reducing introducing sentences of each paragraph and some extra explanation to make it more compact.

2) *Fig 5 D. I am not sure about the nomenclature here. The second panel is a clear bipolar clustered figure (not a pseudo bipolar), the third one I would called it a pseudo-bipolar spindle. The extra pole is within the main spindle.*

> It is true that the spindle that this reviewer pointed out is smaller than the main two spindles. However, there is a significant distance between the small spindle and main ones, and, in addition, the small spindle is oriented towards different direction. Therefore and since such case was frequently found in the experiments, we decided to define this as an extra spindle.

Acceptance

09 October 2014

Thank you for submitting your revised manuscript for our consideration.

It has now been seen once more by all three original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Referee #1

The authors have done a very good job with the revised version of their manuscript. They included new data as requested and made the necessary modifications and clarifications I requested. Although some issues were not addressed for technical reasons, I am supportive for publication of the current version in EMBO.

Referee #2

The authors have adequately addressed my major concerns in their revised manuscript. I am now happy to recommend it for publication in EMBO J.

Referee #3

Slight modification to the title: RBM14 prevents assembly of centriolar protein complexes to maintain mitotic spindle integrity

The revised version of the article by Shiratsuch and colleagues has addressed all my comments. I think this is a novel study that changes our view on non canonical centriole duplication and identifies RBM14 in this process. I would recommend publication by EMBO journal. I would advise, however the authors to try to simplify the text and to clearly state their main messages. There has been an important effort already that it is easily recognized between this version and the previous one. However, I think that it is still difficult for the reader to understand the text sometimes. In addition, I am not sure about the terminology chosen by the authors to describe the structures they identified. I find it very confusing. The model in Figure 7 could also be improved. The 3 points suggest almost that there are un-related.