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A TACC3/ch-TOG/clathrin complex stabilises kinetochore fibres by inter-microtubule bridging

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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 23 September 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three express interest in your study, but all three raise a number of concerns that would need to be addressed before we could consider publication in the EMBO Journal. Their reports are explicit and therefore I don't need to go into detail, but I would draw your attention to a couple of critical points. Firstly, referee 2 (point 1) finds that the data regarding the order of recruitment of CHC, TACC3 and chTOG needs to be improved - particularly in terms of showing the efficacy of knock-down, as well as by looking at endogenous proteins. Secondly, both referees 1 and 2 (ref1 point 4, ref2 point 6) request analysis of the effects of TACC3 and/or chTOG depletion on inter-MT bridges; while I do recognise that such analysis would require substantial work, I agree with the referees that it would substantially strengthen your model and would therefore strongly encourage you to follow this suggestion.

Given the positive assessment of the referees, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers (especially, but not limited to those outlined above). I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as

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soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension. Also, please don't hesitate to get in touch if you have any questions or comments about your revision.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors explore the relationship between Clathrin Heavy Chain (CHC), TACC3, and TOG in spindles. They verify a complex among these proteins in mitotic cells and present evidence that TACC3 and TOG work to recruit CHC to spindles. They go on to use electron microscopy to show that this complex participates in forming inter-MT cross bridges in kinetochore fibers. Loss of CHC reduces the number of MTs in kinetochore fibers and eliminates the shortest of a series of MT cross links in spindles.

Overall, the work presented here is carefully done and some of the data would substantially add to the field. The strength of the manuscript lies in the quantitative electron microscopy that demonstrates that this protein complex participates in cross linking spindle MTs. That is an important advance that adds insight into both the role of CHC in spindles and how it works with TACC3 and TOG for that purpose.

The weakness of the manuscript is the heavy emphasis on the hierarchy for how these proteins recruit each other to spindles. There is obviously controversy in how this works, and the authors provide some compelling data that TACC3 and TOG recruit CHC to spindles and not vice versa as shown by other groups. Notably, the fact that CHC does not bind MTs fits the interpretation presented here. Nevertheless, a much stronger manuscript could be built if the authors focused their attention on the more important aspects of the work that include the EM work showing that this complex plays a role in cross linking MTs. It is appropriate to mention the controversy to raise the issue, but don't focus on it as a major element of the work as it is done in the current draft.

Specific points to be addressed:
1. The statement on page 5 from Figure 2A that there is "dramatically reduced" localization of CHC to spindles lacking TACC3 or TOG seems too strong. The histograms show ~33% reductions making the term "dramatically" seem too strong in my opinion.

2. I think the model in panel 4D needs to be moved to the end and be discussed in a more speculative manner. I think the model is strongest after the EM data is included in the overall evaluation of the role of CHC in spindles.

3. The arm length of the CHC triskelion is slightly shorter than the cross bridge length measured or the class of cross bridge that is lost upon CHC depletion. The authors need to comment on that and include more about what is known about CHC structure into their discussion and model.

4. Based on the model proposed, loss of TACC3 or TOG should also eliminate that short group of cross bridges because CHC should be lost under those conditions. I realize this would take a lot of work to do by EM, and that would be optimal. However, perhaps they could try to develop a quantitative IF assay to measure MT densities in spindles under these different conditions (maybe with or without calcium or cold pretreatment to eliminate non-kinetochore MTs). The IF images presented in figure 2 don't offer much insight into this.

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5. The methods did not clearly explain how MTs were identified as part of kinetochore fibers. How was this done? Also, was the loss of spindle MTs specific to kinetochore fibers or was the non-kinetochore fiber class of MTs also reduced upon CHC depletion? It would be quite striking and important if this complex specifically cross links MTs in kinetochore fibers and not among all other spindle MTs.

Referee #2 (Remarks to the Author):

Review of Booth et al A TACC3/ch-TOG/clathrin complex stabilises kinetochore fibres by inter-microtubule bridging

In this manuscript Booth and colleagues propose and test a hypothesis regarding the role of a molecular complex comprising clathrin/TACC3/ch-TOG in cross-linking kinetochore fibres. This protein complex has been recently identified by 2 other groups, but this is the first study to assign a specific function to the complex. While the hypothesis is interesting and the manuscript represents a substantial amount of work, currently one needs imagination and good will to believe the model proposed.

The manuscript is well written and the data is explained clearly and in a concise manner. The Abstract and Discussion are both informative.

Major points:
1. The authors disagree with recent findings as to whether Clathrin targets TACC3 (suggested by previous work) or vice versa (this study). While it is important to establish whether these publications are wrong about the order of these targeting events, the data presented in Fig. 2 is not convincing enough to refute previous findings. While the authors claim that their shRNA's work well, there are no Western blots or immunofluorescence data to substantiate their statement. Also, there is little information on the duration of shRNA expressions/experiments. Since Clathrin and ch-Tog antibodies appear to be specific I struggle to see why GFP-tagged proteins are used for quantitations instead of the antibodies against endogenous proteins. I also wonder why localisation of GFP-tagged proteins differs from the antibody stainings (Fig. 2); one would expect the antibodies to react with the overexpressed protein. Finally, current data analysis in Fig. 2B,D,F does not take it into account that microtubule density is not uniform in the various knockdowns.
2. The shRNA phenotypes shown in Figs. 2 and 3 do not seem to be consistent (e.g. ch-TOG shRNA gives multipolar spindles in Fig. 2 and nice bipolar ones in Fig. 3). Or is it possible that TACC3 overexpression rescues spindle formation in ch-TOG-depleted cells (Fig. 3D, last panels)? In addition, based on Fig. 3D, spindle localisation of GFP-ch-TOG is independent of TACC3 , but in Fig. 2C the authors claim that TACC3 siRNA reduces this pool of GFP-ch-TOG. These inconsistencies must be resolved or at least discussed. Incomplete shRNA is a likely culprit as it is unclear how shRNA efficiencies were assessed in cells that were transfected with two different shRNA's as well as a GFP construct. Pictures of shRNA-transfected cells with relevant antibodies or Western blots should be shown in Figure or Supplementary information.
3. I am confused by the findings in Fig. 4C. GFP-TACC3 S558A does not localise to the spindle, but then why does it prevent Clathrin recruitment to the spindle in the presence of endogenous TACC3? Is it possible that TACC3 S558A binds clathrin in the cytosol? This point could be tested experimentally.
4. Fig. 5 shows an interesting approach, but it is difficult to judge from the examples that the cross-sections truly represent kinetochore microtubules, especially in left panels where position of the kinetochore is not shown. Have the authors considered including Nuf2/Hec1 siRNA as a negative control (to abolish k-fibres)? If this is beyond the scope of this study, I would recommend the authors to include more details/pictures of serial sections in supplementary material as well as lower magnification EM pictures in figures to aid orientation.
5. Pictures of inter-microtubule bridges are rather faint, but are probably visible to the trained eye. In lower left panel of Fig. 6A microtubules show extensive bending between the bridges. I was not aware of such patterns; are they common? The authors argue that short bridges disappear when CHC is depleted, but it is difficult to exclude the possibility that changes in bridge frequency and length in CHC shRNA are merely a consequence of destabilisation (or lower numbers) of kinetochore fibres. This should be discussed in the manuscript.
6. Have the authors tried to see if depletion of TACC3 or ch-TOG give similar results? Based on the

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model in Fig. 4, one would expect TACC3 depletion to give the strongest phenotype, since it's upstream of Clathrin. Such data would really substantiate the model.
7. Images in Figure 8 are too small to appreciate. The distribution of gold particles should be shown in the context of a whole cell as in A, but at higher magnification. I find it surprising that there are so few k-fibres in the spindle and also that they are not coated with GFP-tubulin-specific gold. Based on this image it is difficult to appreciate if gold particles are enriched in the spindle area at all. Also, is it possible that top row of images represent clathrin and bottom row are the tubulin ones? The gold appears smaller in those. Or else the magnification is different for these images.
8. It would be more logical to include Fig. 6C in Fig. 7 instead.

Referee #3 (Remarks to the Author):

This manuscript addresses the question of clathrin function in the mitotic spindle. Clathrin was previously shown to have a role in spindle function (Royle 2005), specifically in stabilizing kinetochore-microtubule fibers. This paper adds mechanistic insight to the previous observations. A complex of clathrin with the MAPs TACC3 and ch-TOG is purified from mitotic spindles. As these two MAPs are known to be spindle associated, the characterization of the complex provides a nice explanation for how clathrin is targeted to spindle microtubules. RNAi and overexpression experiments suggest a model in which TACC3 binds microtubules and recruits TOG and clathrin. The whole process depends on phosphorylation of TACC3, a known substrate of Aurora A kinase. EM analysis shows that kinetochore fibers have fewer microtubules in clathrin-depleted cells, and the microtubules are spaced farther apart. Furthermore, inter-microtubule bridges can be detected by EM, and a subset of these bridges are lost in clathrin-depleted cells. Finally, immunogold labeling shows that the bridges are associated with clathrin.

The paper potentially makes a significant contribution to our understanding of how kinetochore microtubules are stabilized by clathrin. The inter-microtubule bridges, in particular, are an intriguing area that has not been well explored. However, there are issues with the data presentation and analysis that need to be addressed to make the interpretation convincing.

Comments:

"Topology" doesn't seem like the right word for the analysis of the TACC/TOG/clathrin complex. Statements like "clathrin is placed most distal to the microtubule" (p. 8) and "clathrin sits at the most exterior aspect of the complex" (p. 11) are not supported by the data and would require some other structural analysis.

The criteria for what constitutes an inter-MT bridge in the EM analysis are not clear. Since these structures have not been studied in the recent literature, it would be helpful to define the term more precisely and to describe in more detail what kinds of structures are observed. These bridges are a key part of the paper.

It is not clear what this statement means: "54.2% of clathrin label was specific to inter-MT bridges" (p. 9). What are the criteria for this measurement?

Figure 1: the F1-F7 labels are not explained. It is difficult to interpret the figure without understanding the notation. Also, the legend for panel E states that anti-myc was used as a control, but it's not clear what data represent the control as opposed to the anti-clathrin antibody.

Figures 3 and 4 need some quantitation so that the interpretation does not rely on single images.

Figure 7: the insets are too small and difficult to see. It's not clear what "normalized frequency" means in panel B. It's not convincing that there are multiple distinct peaks in the histograms in panel B. It's not clear what the "Multi-peak analysis" means, or how the curves fitting the two distributions were created. Overall, the analysis in this figure is quite opaque.

Figure 8: The data presentation in this figure is generally poor. The legend for panel B says that tubulin and clathrin are labeled with 5 nm and 10 nm gold, respectively, but the panels labeled clathrin include both large (top rows) and small (bottom row) gold particles. Also, it's not clear

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where the bridges are. The panels need some additional visual aids and may need to be larger. Overall, panel B is very confusing and difficult to interpret. In panel C it is difficult to relate the measurements to the data in panel B. Clearly labeled examples of primary data are essential to make the analysis convincing. Also, the way the two overlapping histograms are presented is difficult to visualize; there are better ways to present the data. In panel E, it's not clear what "distances are relative to the origin" means. A schematic showing how the analysis was done would be helpful. Finally, I couldn't find the scale bar in this figure.

1st Revision - authors' response 08 December 2010

Referee #1

We thank the referee for their time, their careful consideration of our manuscript and for their constructive criticism.

A much stronger manuscript could be built if the authors focused their attention on the more important aspects of the work that include the EM work showing that this complex plays a role in cross linking MTs. It is appropriate to mention the controversy to raise the issue, but don't focus on it as a major element of the work as it is done in the current draft.
We appreciate that the first draft did dwell on the order of recruitment and we're glad that the referees were convinced by our view of how the TACC3/ch-TOG/clathrin complex is recruited to MTs. In light of the corrections described below, the manuscript is now weighted much more towards the EM and the inter-MT bridges, as the referee suggested.

1. The statement on page 5 from Figure 2A that there is "dramatically reduced" localization of CHC to spindles lacking TACC3 or TOG seems too strong. The histograms show ~33% reductions making the term "dramatically" seem too strong in my opinion.
This is a misunderstanding. The data in the bar charts are normalized so that a value of 1 (dotted line) represents no detectable recruitment. The reduction from ~1.5 to ~1 is dramatic: it cannot get any lower. It is not a reduction of ~33% but of ~90%. We have added a note to the figure legend to make this clear and the reduction is stated in the results section.

2. I think the model in panel 4D needs to be moved to the end and be discussed in a more speculative manner. I think the model is strongest after the EM data is included in the overall evaluation of the role of CHC in spindles.
This is a great suggestion and we have moved the model to become Supplementary Figure 6.

3. The arm length of the CHC triskelion is slightly shorter than the cross bridge length measured or the class of cross bridge that is lost upon CHC depletion. The authors need to comment on that and include more about what is known about CHC structure into their discussion and model.
We have added a discussion of the relative sizes of bridges and triskelia in detail in the manuscript. This is now much easier to do following the referee's suggestion to move the model figure to the end of the paper. The leg length of clathrin is actually longer than the class of bridge that is lost upon CHC depletion. We now comment on this and describe in more detail the relative sizes shown in the model figure.

4. Based on the model proposed, loss of TACC3 or TOG should also eliminate that short group of cross bridges because CHC should be lost under those conditions. I realize this would take a lot of work to do by EM, and that would be optimal. However, perhaps they could try to develop a quantitative IF assay to measure MT densities in spindles under these different conditions (maybe with or without calcium or cold pretreatment to eliminate non-kinetochore MTs). The IF images presented in figure 2 don't offer much insight into this.
We have now carried out an EM analysis of inter-MT bridges in TACC3-depleted cells. This analysis revealed a decrease in inter-MT bridges that was similar to that observed in clathrin-depleted K-fibres (Figure 9). Importantly, the shortest bridges are again missing (Figure 10). This substantially strengthens our model that TACC3 and clathrin are components of the same (shortest) form of inter-MT bridge in K-fibres.
We also looked at ch-TOG-depleted K-fibres by EM. However, the fibres were very disorganized

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and we found that our current analysis methods could not be applied reliably. This likely reflects roles of ch-TOG outside out of inter-MT bridges. This has been added to the results and is included as Supplementary Figure 5.

5. The methods did not clearly explain how MTs were identified as part of kinetochore fibers. How was this done?
This is an important point and we thank the referee for pointing out that this was unclear. We have now improved the paper by a more extensive description of K-fibre identification in the Methods section.
Essentially, K-fibres were identified in two ways. In longitudinal sections (bridge quantifications) the K-fibres were easily identified as the fibre could be seen to terminate at a kinetochore. In orthogonal sections (MT quantifications) some K-fibres were followed to the kinetochore by serial sectioning, but this was not feasible for all K-fibres. To ensure that we only analysed K-fibres, we limited our orthogonal sections to within 1 micron of the chromosome. In this region, non-kMTs have very few interactions with kMTs (McDonald *et al*, 1992) and so should not interfere with our analysis. Also, reconstructions of interpolar MTs in metaphase cells show only bundles of 2-6 MTs (Mastronarde *et al*, 1993) and we only quantified bundles of >6 MTs. This means that we may have underestimated the effect of clathrin-depletion but we are certain that we have only analysed K-fibres.
Also, was the loss of spindle MTs specific to kinetochore fibers or was the non-kinetochore fiber class of MTs also reduced upon CHC depletion? It would be quite striking and important if this complex specifically cross links MTs in kinetochore fibers and not among all other spindle MTs.
We think that this complex does specifically cross-link kMTs, but demonstrating this is tough. Reliably identifying ipMTs is difficult due to the small size of the fibres at metaphase. At anaphase, the bundles are much larger (Mastronarde *et al*, 1993), however clathrin is absent from these fibres. This is a very interesting question that the referee has raised and it is one that can be addressed in the future.

Referee #2

We thank the referee for their time, their careful consideration of our manuscript and for their constructive criticism.

1. The authors disagree with recent findings as to whether Clathrin targets TACC3 (suggested by previous work) or vice versa (this study). While it is important to establish whether these publications are wrong about the order of these targeting events, the data presented in Fig. 2 is not convincing enough to refute previous findings. While the authors claim that their shRNA's work well, there are no Western blots or immunofluorescence data to substantiate their statement. Also, there is little information on the duration of shRNA expressions/experiments.
Our statement is now substantiated by quantification of depletion of the endogenous proteins in Supplementary Figure 2. We found western blotting to be difficult to interpret due to the relatively low transfection efficiency of pBrain plasmids (Supplementary Figure 2C, D). However, our immuofluorescence quantifications indicate that our shRNAs work well. The details of the duration of shRNA expression are now included more prominently in the paper.

Since Clathrin and ch-Tog antibodies appear to be specific I struggle to see why GFP-tagged proteins are used for quantitations instead of the antibodies against endogenous proteins. I also wonder why localisation of GFP-tagged proteins differs from the antibody stainings (Fig. 2); one would expect the antibodies to react with the overexpressed protein.
This is a misunderstanding: GFP-tagged proteins were not used for quantifications. Our pBrain plasmids co-express a fluorescent reporter alongside shRNA. The GFP was simply shown to indicate that the cell was targeted for RNAi. This has obviously caused confusion and so we have removed the panel from Figure 2.
The original Figure 2 showed a quantification of spindle localisation of endogenous proteins following RNAi, but the pictures for clathrin and ch-TOG used an RFP tagged protein to illustrate the change in localisation. We recognise that this was too confusing and so Figure 2 has been improved by only showing endogenous protein localisation.

Finally, current data analysis in Fig. 2B,D,F does not take it into account that microtubule density

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is not uniform in the various knockdowns.
We have now re-analysed the images from these experiments and we have improved Figure 2 by adding the normalised MT density recorded in the spindle ROI. To allow comparison, the data are now presented in the style of the recent JCB paper on the TACC3-clathrin interaction (Lin *et al*, 2010). We found that the changes in MT density cannot account for the observed changes in spindle localisation of complex components.

2. The shRNA phenotypes shown in Figs. 2 and 3 do not seem to be consistent (e.g. ch-TOG shRNA gives multipolar spindles in Fig. 2 and nice bipolar ones in Fig. 3).
Knockdown of ch-TOG gives a range of phenotypes from multi-polar spindles to disorganised but bipolar spindles. This is previously described (Gergely *et al*, 2003) and can also be seen in Supplementary Figure 5. We agree with the referee that we should have picked only bipolar spindles for presentation to allow for easy comparison and we have now improved the figure accordingly.

Or is it possible that TACC3 overexpression rescues spindle formation in ch-TOG-depleted cells (Fig. 3D, last panels)? In addition, based on Fig. 3D, spindle localisation of GFP-ch-TOG is independent of TACC3 , but in Fig. 2C the authors claim that TACC3 siRNA reduces this pool of GFP-ch-TOG. These inconsistencies must be resolved or at least discussed. Incomplete shRNA is a likely culprit as it is unclear how shRNA efficiencies were assessed in cells that were transfected with two different shRNA's as well as a GFP construct. Pictures of shRNA-transfected cells with relevant antibodies or Western blots should be shown in Figure or Supplementary information.
We felt that the experiments summarised in Figure 3D posed more questions than they answered. We agree that more work is needed to identify why GFP-ch-TOG can be recruited to MTs in the absence of TACC3 and also to determine the extent of double knockdown. As part of the revision process, the paper has grown considerably in size and also Referee #1 suggested that we should focus much less on the minutiae of complex recruitment to MTs. For these reasons, we have decided to remove the reciprocal knockdown experiments that were shown in Figure 3D.

3. I am confused by the findings in Fig. 4C. GFP-TACC3 S558A does not localise to the spindle, but then why does it prevent Clathrin recruitment to the spindle in the presence of endogenous TACC3? Is it possible that TACC3 S558A binds clathrin in the cytosol? This point could be tested experimentally.
We think that clathrin recruitment is via endogenous TACC3 and that expression of TACC3(S558A) removes the endogenous TACC3 from the spindle. We have now included two extra pieces of data in support of this view. First, the GFP-TACC3 WT or S558A expression experiment was also carried out on a background of TACC3-depletion (Fig 4D). Second, we have tried to examine the removal of endogenous TACC3 on the spindle in cells expressing GFP-TACC3(S558A) and find that the levels are reduced. However, we cannot formally exclude the possibility that TACC3(S558A) can bind to clathrin. The recent TACC3-clathrin interaction papers indicate that *in vitro* this interaction requires S558 (Fu *et al*, 2010; Lin *et al*, 2010). As to whether any TACC3-clathrin interactions occur in the cytosol, we are unable to verify the interaction as described in the recent papers and have only identified TACC3/ch-TOG/clathrin complexes on purified spindles and not in cytosol.

4. Fig. 5 shows an interesting approach, but it is difficult to judge from the examples that the cross-sections truly represent kinetochore microtubules, especially in left panels where position of the kinetochore is not shown. Have the authors considered including Nuf2/Hec1 siRNA as a negative control (to abolish k-fibres)? If this is beyond the scope of this study, I would recommend the authors to include more details/pictures of serial sections in supplementary material as well as lower magnification EM pictures in figures to aid orientation.
The question is: how can we be certain that we are analysing K-fibres? This question was also asked by Referee #1. This is an important point and we have now improved the paper by a more extensive description of K-fibre identification in the Methods section.
Essentially, K-fibres were identified in two ways. In longitudinal sections (bridge quantifications) the K-fibres were easily identified as the fibre could be seen to terminate at a kinetochore. In orthogonal sections (MT quantifications) some K-fibres were followed to the kinetochore by serial sectioning, but this was not feasible for all K-fibres. To ensure that we only analysed K-fibres, we limited our orthogonal sections to within 1 micron of the chromosome. In this region, non-kMTs have very few interactions with kMTs (McDonald *et al*, 1992) and so should not interfere with our analysis. Also, reconstructions of interpolar MTs in metaphase cells show only bundles of 2-6 MTs

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(Mastronarde *et al*, 1993) and we only quantified bundles of >6 MTs. This means that we may have underestimated the effect of clathrin-depletion but we are certain that we have only analysed K-fibres.

5. In lower left panel of Fig. 6A microtubules show extensive bending between the bridges. I was not aware of such patterns; are they common?
Using chemical fixation, MTs do undergo some bending (Mastronarde *et al*, 1993; McDonald *et al*, 1992). High-pressure freezing improves MT preservation, but this is not compatible with our current CLEM approach. The MTs did not bend extensively, i.e. move out of section, which would prevent accurate quantification. The degree of MT bending and the morphology of inter-MT bridges is identical to that observed in a classic EM study of inter-MT bridges (Hepler *et al*, 1970).

The authors argue that short bridges disappear when CHC is depleted, but it is difficult to exclude the possibility that changes in bridge frequency and length in CHC shRNA are merely a consequence of destabilisation (or lower numbers) of kinetochore fibres. This should be discussed in the manuscript.
We share the Referee's view that the relationship between change in MT number and changed bridge frequency is difficult to define. After all, inter-MT bridges by definition must occur between MTs and so changes in either MTs or bridges will likely affect the other. This is an important point and we addressed this in the manuscript by analysing the bridge frequency per unit length of paired MT. This allowed us to look at loss of bridges in those regions where bridging can occur; in isolation from the changes in MT number and spacing that occur following clathrin-depletion (and presumably following TACC3-depletion). We found that bridge loss occurs even in these regions (Figures 6B and 9B). This suggests that bridge loss causes the loss of MTs and not the other way around.

6. Have the authors tried to see if depletion of TACC3 or ch-TOG give similar results? Based on the model in Fig. 4, one would expect TACC3 depletion to give the strongest phenotype, since it's upstream of Clathrin. Such data would really substantiate the model.
We have now carried out an EM analysis of inter-MT bridges in TACC3-depleted cells. This analysis revealed a decrease in inter-MT bridges that was similar to that observed in clathrin-depleted K-fibres (Figure 9). Importantly, the shortest bridges are again missing (Figure 10). This substantially strengthens our model that TACC3 and clathrin are components of the same (shortest) form of inter-MT bridge in K-fibres.
We also looked at ch-TOG-depleted K-fibres by EM. However, the fibres were very disorganized and we found that our current analysis methods could not be applied reliably. This likely reflects roles of ch-TOG outside out of inter-MT bridges. We have included this result as Supplementary Figure 5.

7. Images in Figure 8 are too small to appreciate. The distribution of gold particles should be shown in the context of a whole cell as in A, but at higher magnification. I find it surprising that there are so few k-fibres in the spindle and also that they are not coated with GFP-tubulin-specific gold. Based on this image it is difficult to appreciate if gold particles are enriched in the spindle area at all. Also, is it possible that top row of images represent clathrin and bottom row are the tubulin ones? The gold appears smaller in those. Or else the magnification is different for these images.
We have improved the Figure 8 in the following ways:
1. Added an 'intermediate zoom' picture of tubulin gold label in a K-fibre to show the location of the panels in the upper part of B. This shows the location of some immunogold label in the context of a whole K-fibre. Note, that it is not possible to see 10 nm gold at lower magnification as the referee requested.
2. Improved the scatter plot to show a colour-coded plot of all data and a zoom of the area of interest. The legend has been improved to be clear about the distances that were measured.
3. Presented the histograms as separate plots rather than overlays.
4. Added a schematic diagram (G) that shows the relative position of the median distances of tubulin and clathrin gold on a scale diagram of two MTs connected by an idealised bridge.
5. A scale bar has been added to part A.
We now explain the preparation of samples more clearly as this was a cause of misunderstanding for this Referee and Referee #3. We have not labelled for clathrin and tubulin together in the same sample. Cells expressed *either* GFP-tubulin *or* GFP-clathrin. The spindles were labelled with two primary antibodies to maximise labelling: *either* anti-GFP and anti-tubulin *or* anti-GFP and anti-

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clathrin. The antibodies were detected with 5 nm and 10 nm gold conjugated secondary antibodies in both cases. We now show examples of either type of labelling in part B.
Finally, the number of K-fibres that can be visualised in a 150 nm section by EM *is* fewer than can be seen in something like a confocal section (~400 nm). Also, the density of our immunogold labelling was low due to the stringent conditions we used, but it is very specific. The inset plot of all data shows the level of non-specific labelling i.e. gold that is not near to MTs or bridges and it is only 1-2% of the total label.

8. It would be more logical to include Fig. 6C in Fig. 7 instead.
This is an excellent suggestion and we have now done this.

Referee #3

We thank the referee for their time, their careful consideration of our manuscript and for their constructive criticism.

"Topology" doesn't seem like the right word for the analysis of the TACC/TOG/clathrin complex. Statements like "clathrin is placed most distal to the microtubule" (p. 8) and "clathrin sits at the most exterior aspect of the complex" (p. 11) are not supported by the data and would require some other structural analysis.
We have changed these terms in the text. Instead of topology we talk about order of recruitment.

The criteria for what constitutes an inter-MT bridge in the EM analysis are not clear. Since these structures have not been studied in the recent literature, it would be helpful to define the term more precisely and to describe in more detail what kinds of structures are observed. These bridges are a key part of the paper.
We have added some details to the results and methods section to clarify this issue.

It is not clear what this statement means: "54.2% of clathrin label was specific to inter-MT bridges" (p. 9). What are the criteria for this measurement?
The criterion is a gold particle within 16 nm of a bridge. 16 nm is the maximum distance that a gold particle can be from a structure that it is labelling (Ottersen, 1989). The definition was in the paper but buried in the Methods. We have now made this clearer.

Figure 1: the F1-F7 labels are not explained. It is difficult to interpret the figure without understanding the notation. Also, the legend for panel E states that anti-myc was used as a control, but it's not clear what data represent the control as opposed to the anti-clathrin antibody.
We have made several changes to Fig 1 and its legend to make it clear that F1-F7 are the fraction numbers collected during the spindle purification. We have improved the presentation of the mass spec experiment shown in the table to include the control antibody results.

Figures 3 and 4 need some quantitation so that the interpretation does not rely on single images.
We have now quantified the images from these figures and present the data alongside the images. These analyses strengthen substantially our conclusion that TACC3 is the primary recruitment factor for the complex. The analysis also revealed that expression of ch-TOG did not significantly increase the amount of clathrin or TACC3 at the spindle.
We have removed the reciprocal knockdown experiments that were shown in Figure 3D. These experiments were peripheral to the main focus of the paper, which, as referee #1 suggested should be focused more on the inter-MT bridges and not so much on the minutiae of the recruitment of the complex to MTs. Secondly, following quantitation, the results were difficult to interpret.

Figure 7: the insets are too small and difficult to see. It's not clear what "normalized frequency" means in panel B. It's not convincing that there are multiple distinct peaks in the histograms in panel B. It's not clear what the "Multi-peak analysis" means, or how the curves fitting the two distributions were created. Overall, the analysis in this figure is quite opaque.
We are grateful to the referee for the feedback on this figure. We were uncertain as to how to analyse these data for the first draft and it is fair to say that we could have been clearer about how the analysis was done. As part of the revisions we have now analysed bridge lengths in control and TACC3-depleted cells. This analysis, which revealed populations of bridges that are broadly similar

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in lengths to the previous dataset, has given us more confidence that our multi-peak analysis is sound.
Figure 7 and its results section have now been improved in the following ways:
1. Separated large histograms are shown at 2 nm resolution with the multi-peak fits overlaid.
2. The normalisation of the frequency data was abandoned as it made no difference to the multi-peak fit and obviously was just confusing the presentation unnecessarily.
3. We are now explicit about how the fits were done and have included a Supplementary Figure to "show our working".
4. Added a statement to describe that multi-peak fits were a better description of the datasets compared to a single curve.
Figure 10 (TACC3-depletion data) is laid out in a similar way to Figure 7.

Figure 8: The data presentation in this figure is generally poor. The legend for panel B says that tubulin and clathrin are labeled with 5 nm and 10 nm gold, respectively, but the panels labeled clathrin include both large (top rows) and small (bottom row) gold particles. Also, it's not clear where the bridges are. The panels need some additional visual aids and may need to be larger. Overall, panel B is very confusing and difficult to interpret. In panel C it is difficult to relate the measurements to the data in panel B. Clearly labeled examples of primary data are essential to make the analysis convincing. Also, the way the two overlapping histograms are presented is difficult to visualize; there are better ways to present the data. In panel E, it's not clear what "distances are relative to the origin" means. A schematic showing how the analysis was done would be helpful. Finally, I couldn't find the scale bar in this figure.
We have improved the Figure 8 in the following ways:
1. Added an 'intermediate zoom' picture of tubulin gold label in a K-fibre to show the location of the panels in the upper part of B (after the suggestion of Referee #2).
2. The bridges that are visible in the gallery in B have been marked out by arrows.
3. Improved the scatter plot to show a colour-coded plot of all data and a zoom of the area of interest. The legend has been improved to be clear about the distances that were measured.
4. Presented the histograms as separate plots rather than overlays.
5. Added a schematic diagram (G) that shows the relative position of the median distances of tubulin and clathrin gold on a scale diagram of two MTs connected by an idealised bridge.
6. Added a scale bar to part A.
We now explain the preparation of samples more clearly as this was a cause of misunderstanding for this referee and Referee #2. We have not labelled for clathrin and tubulin together in the same sample. Cells expressed *either* GFP-tubulin *or* GFP-clathrin. The spindles were labelled with two primary antibodies to maximise labelling: *either* anti-GFP and anti-tubulin *or* anti-GFP and anti-clathrin. The antibodies were detected with 5 nm and 10 nm gold conjugated secondary antibodies in both cases. We now show examples of either type of labelling in part B.

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2nd Editorial Decision 04 January 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-75783R to the EMBO Journal. It has now been seen again by referees 2 and 3, whose comments are appended below. As you will see, both referees find the manuscript to be significantly improved and are fully supportive of publication, but referee 2 has a few remaining concerns that need to be addressed first. The major concern - as to how you can identify depleted cells for EM analysis - is an important one, and it needs to be clear how you are able to do so (or, if you can not, how you can be confident in the significance of your results). I would also agree with the referee that much of the text in the figure legends is too small to be read easily, and would therefore ask you to amend the figures to increase font size where necessary.

I hope that these final changes should be relatively straightforward; once we have the revised manuscript, we should then be able to accept it for publication without further delay.

Many thanks and best wishes for 2011,

REFEREE REPORTS

Referee #2 (Remarks to the Author):

The manuscript has been improved significantly. The authors considered and satisfactorily addressed most of my comments.

Concern:
Based on the Western blot using endogenous antibodies in Suppl. Fig.2 the shRNA efficiency appears rather poor (in the range of 50-60%). The reporter shown on the left of this figure suggests that it is the uptake of the construct that is limiting (for instance there is only one GFP-positive cell in ch-TOG shRNA treated field). How do the authors know whether a cell analysed for EM is depleted? Is the multi-peak analysis for bridge length statistically meaningful if only half of the cells are actually depleted? These are iportant points to consider when analysing and describing the data and should be discussed in the manuscript.

Minor points:

1. On p. 6 last sentence of first paragraph does not read correctly (....following depletion of TACC3 and ch-TOG others...)
2. On p.9 and 10. Multi-peak analysis of clathrin and TACC3-depleted cells show the absence of short bridges. However, the control populations described are different for the 2 experiments (control on p9; 15, 21, 30, 53nm vs. control on p10; 17, 24, 50nm). This raises the possibility that the distribution of bridge sizes is not strongly reproducible between experiments or that there is a proportion of cells that are not depleted of TACC3 or Clathrin among the shRNA population (see concern above). This should be clarified and discussed.
3. I would recommend simplifying Fig. 4D by including only the non-siRNA results in the graph and moving the siRNA data to a Supplementary Figure instead.
4. Parts of some figures have very small font sizes particuarly in print; these should be adjusted.
5. In Figure 1 CHC and clathrin RNAi are both used.

Referee #3 (Remarks to the Author):

My concerns have been addressed.

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2nd Revision - authors' response 05 January 2011

Thank you very much for your positive decision e-mail of 4th January. We have now tidied up the manuscript in response to the remaining niggles of Referee #2, see below. We are grateful to all the referees for their time and for a constructive peer review process overall.

- Our ultrastructural analysis uses two technologies to ensure that we only analyse cells that have undergone RNAi. The first is the pBrain vector system that co-expresses GFP and shRNA, meaning that cells that have undergone RNAi are green. The second is the use of correlative light-electron microscopy (CLEM). This means that we can find a green cell (at the right stage of mitosis) by light microscopy and then locate the same cell later by EM (an example is shown in Supp. Fig. 5). The referee has understood the first technique but not the second. We are mortified that we didn't manage to get these ideas across clearly to the referee, especially given how laborious CLEM is! We have now included a sentence at the beginning of the ultrastructure section to make it clear that it is through the use of CLEM that we are certain that all cells analysed have undergone the appropriate RNAi.
- The multi-peak analyses of control datasets are very similar. The only difference is that the controls for the clathrin RNAi experiments have two peaks with means of 20 and 27 nm whereas the controls for the TACC3 experiments have a single wide peak at 24 nm. The lower number of bridges analysed for the latter (1335 vs 732) means that the resolution is not as good. Consequently, the single wide peak at 24 nm is not seen as two peaks by the multi-peak analysis. We assume that this is the difference that the referee is referring to, rather than the trivial differences in mean lengths (15 v 17 and 53 v 50) which, given the width of the Gaussian distributions, are obviously not significantly different.
- We have not altered Fig. 4D as the referee suggested: it would leave us without an appropriate control on the graph.
- We agree that the font sizes were too small in Figures 1 & 3 and we have now amended the offending panels and made the other minor changes mentioned.

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