Centrosomal ALIX regulates mitotic spindle orientation by modulating astral microtubule dynamics

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1st Editorial Decision 29 August 2017

Thank you again for submitting your manuscript on centrosomal ALIX in spindle orientation to The EMBO Journal, and apologies for its prolonged external evaluation, which was mainly due to limited referee availability at this time of the year. We have now finally received a complete set of reviews, copied below for your information. As you will see, these reports are somewhat ambivalent - on one hand, all referees appreciate the interest and potential importance of your findings, and acknowledge your careful and comprehensive analyses utilizing several different model systems. At the same time, they however also raise a considerable number of well-taken major concerns related to the experimental analyses and their interpretation, which would in our view need to be satisfactorily addressed in order to make this work a strong candidate for EMBO Journal publication.

Although the nature and extent of these concerns indicate that adequately addressing them may require substantial further time and efforts, and may also alter some of the current conclusions, I would in light of the stated overall interest nevertheless like to give you an opportunity to answer to the referees’ criticisms by way of a revised version of the manuscript. Therefore, should you be able to strengthen the present analyses along the lines suggested by the referees, we would remain interested in considering this study further for publication. Since it is our policy to allow only a single round of major revision, I would be happy to discuss a possible extension of our normal three-months revision period - during which time the publication of any competing work elsewhere would have no negative impact on our final assessment of your own study. Furthermore, it may be helpful if you would contact me already during the early stages of your revision with a brief proposal on how you plan to address the various referee points, as well as with any specific questions/comments that you may have related to the reports or this decision.

Please refer to the sections below for additional information on preparing and uploading a revised manuscript.

Thank you again for the opportunity to consider this work for The EMBO Journal. I look forward to
hearing from you in due time.

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REFEREE REPORTS

Referee #1:

In this manuscript, Malerod et al. describe the first characterization of the function of centrosomal ALIX, a protein known to regulate cytokinesis, endosomal biogenesis, virus release and plasma membrane repair in an ESCRT-dependent manner. Previous studies (including one by the authors) already reported that a pool of ALIX is present at the centrosomes in interphase and during cell division. However, the potential role of centrosomal ALIX during cell division was not known. Using a variety of models (neuroblast divisions, germ cell divisions, epithelial cell divisions in Drosophila as well as human Caco2 organoids and HeLa cells), the authors show that ALIX promotes correct spindle orientation by regulating the nucleation of astral microtubules and their stability. The first regulation depends on the recruitment of the MT nucleator gamma-TURC to centrosomes and the second regulation on the recruitment of the MT binding protein MAP1S. Finally, the authors demonstrate that, both in Drosophila and in Human cells, Cep192 recruits ALIX to the PCM.

Overall, this is an excellent study with careful analysis and important conclusions that deserves to be published in the EMBO Journal. The use of several models clearly backs up the conclusion that ALIX contributes to astral microtubule nucleation/stability and thus to spindle orientation. I have nevertheless a few questions that should be addressed before recommending publication.

Major points

1- All the presented data converge towards a role of ALIX at the centrosome in spindle orientation. However, ALIX has many other functions outside the centrosome. For instance, ALIX could regulate endosomal trafficking, which could indirectly control spindle orientation. It would be therefore important to deplete ESCRT components (e.g. CHMP4B) and demonstrate that there is no spindle orientation defects in these conditions. The Reviewer does not ask to test this in all presented models, but in the most convenient one (e.g. Caco2 organoids or HeLa cells in culture).

2- The part regarding MAPS1 is the only section of the manuscript which is not entirely convincing. In particular the conclusion based on Fig. 7E that ALIX regulates the recruitment of MAP1S is not really significant (74% vs. 84% with no quantification of the MAP1S levels upon ALIX depletion). In addition, results from Figure 1F are interesting but, in my opinion, do not support the fact that ALIX recruits MAP1S but rather that MAP1S can stabilize MTs independently from ALIX. Indeed, if ALIX recruited MAP1S, why MAP1S could rescue the instability of MTs in the absence of ALIX? The results instead argue for two independent pathways.

I therefore suggest the two following experiments to backup this part of the manuscript:
1) Does the overexpression of any MAPs rescue the defects in astral MT length after ALIX depletion? If it were the case, the authors should revise the conclusion of the MAP1S section.
2) Does ALIX affect the endogenous MAP1S staining on centrosomes/spindle?

Dallol et al. and Tegha-Dunghu et al. provide nice pictures of endogenous MAP1S. This experiment would more directly answer whether ALIX regulates MAP1S recruitment to the spindle (or specifically to the centrosomes for instance).

3- The specificity of the ALIX staining should be demonstrated. Does ALIX signal at the centrosome/PCM disappear in Drosophila Alix1 mutant and in ALIX-depleted HeLa cells?

Minor points

1- Does ALIX regulate MT nucleation also in interphase cells? The authors could analyze their data
for instance in human interphase cells, as shown in Figure 5D.

2- For spindle orientation analysis (N=3 experiments, Figure 3A, 4A, 4D etc.), the authors should provide error bars.

3- It would be interesting to measure the speed of spindle movement after ALIX depletion in at least one model. According to the conclusion of the manuscript, one should expect slow rotation of the spindles in depleted cells.

Referee #2:

In the paper, Malerod and colleagues investigate the role of ALIX at centrosomes in both Drosophila and human cultured cells. They provide evidence that ALIX is recruited to centrosomes via an interaction with Spd-2 and show that ALIX is required for the proper recruitment of g-TuRCs to centrosomes and thus for proper centrosomal microtubule nucleation. They also show that ALIX helps recruit MAP1S to microtubules in HeLa cells. They show that various cell types that lack or have reduced levels of ALIX display mitotic spindle alignment defects, in line with the observed reduction in centrosomal microtubules.

Overall the paper is interesting and will be suitable for publication once the authors have addressed the comments below.

1) The authors use structured illumination microscopy (SIM) to examine the sub-centrosomal localisation of ALIX in Figure 1C, but the images look of poor quality, especially as SIM reconstructions are prone to artefactual meshwork patterns. It would be better to perform line or radial plots on confocal images, which are more reliable and have the resolution required to distinguish the PCM from the centrioles. The authors could plot the fluorescence of a centriole marker (e.g. Asl) and ALIX to show that the ALIX fluorescence is more spread out.

2) In figure 2, the authors quantify the number of centrosomes that lack ALIX signal in different mutant backgrounds, but it would be more accurate and useful to quantify the average fluorescence signal from multiple centrosomes from multiple samples (similar to how they quantified g-tubulin and grip 91 levels in Figure 5E). It is strange that some centrosomes would completely lack ALIX, when ALIX can clearly still be recruited in the absence of either Spd-2 or Cnn. A more realistic representation would therefore be the % drop in ALIX centrosomal levels in the different mutants.

3) The authors conclude that ALIX is recruited to centrosomes via a direct interaction with Spd-2, but ALIX can still be recruited in the absence of Spd-2. Thus, although Spd-2 may be the major recruiter of ALIX, ALIX may also be recruited by other means - the authors should discuss this.

4) I would recommend that the authors quantify centrosomal asters in alix clones during prophase. It is very difficult to quantify centrosomal asters in Drosophila neuroblasts during metaphase because the spindle fluorescence is very dominant, even in WT neuroblasts (as can be seen in Figure 5). It is much better to quantify asters during prophase, before the spindle has formed. To do this, they just need to stain for centrioles (Asl) and mitotic DNA (Phospho-histone H3).

5) In Figure EV4A, the asters in the bottom images do not look different between the mutant and the control. Rather, it seems that the control just has a higher contrast.

6) To assess g-tubulin and Grip91 levels at centrosomes in Figure 5E, the authors need to stain with a centriole marker, such as Asl, to be sure that the g-tubulin signal is coming from a centrosome.

7) I have major concerns with the conclusions from the double mutant and double knockdown experiments in Figure 6. The authors use an increase in phenotype in the double mutants/knockdowns to conclude that ALIX also functions independently of g-tubulin. This can only be concluded where the mutants/knockdowns result in a complete absence of protein (e.g. a null mutant). In the blot in Figure 6B, there is still a faint band of g-tubulin, and in our lab we have found that when we immunostain g-tubulin null mutant 3rd instar larval brains for g-tubulin, we can detect g-tubulin at centrosomes in many of the cells. We therefore believe that the maternal contribution of g-tubulin shows strong perdurance, and this fits with the weak phenotypes observed by these authors, ours and others. The presence of protein is also a problem in the RNAi knockdowns in human cells, where the blot in Figure 6D shows that there is plenty of protein left. The increased phenotypes seen in the double mutants/knockdowns could therefore be due to potentiation of the ALIX-g-tubulin pathway i.e. the g-tubulin mutant/knockdown reduces, but does
not abolish, γ-tubulin from the centrosome, and adding in the ALIX mutant/knockdown just makes this worse. I therefore think that the authors need to tone down their conclusions, explaining their data is consistent with a model of separate pathways, but it is not conclusive.

8) I am unsure how spindle orientation can be measured after microtubule depolymerisation in Figure 7?

9) The conclusion that "ALIX controls microtubule stability" (p.17) is too strong. "Influences" would be more accurate.

10) The authors need to present their mass spec data

11) Does the relatively small change in MAPS1 association with microtubules in the absence of ALIX (Figure 7E) correspond to the differences seen in the mass spec?

12) The IPs in Figure 7 were apparently performed under conditions that would keep MTs in the extracts, so any perceived interaction could be due to both MAP1S and ALIX being associated with microtubules. If so, the difference in association between ALIX and different MAPS1 constructs may reflect the difference in the ability of the MAPS1 constructs to bind microtubules.

Minor comments:

a) The authors refer to sequential recruitment of the PCM in their introduction (p.3), and they conclude in their results that ALIX is recruited "relatively early during PCM assembly" (p.7). It is worth noting, however, that the PCM is not assembled sequentially. Instead, PCM components build up together during centrosome maturation i.e. if component A recruits component B, they still build up in levels together; component A does not fully build up before component B. The authors should therefore adjust their wording and conclusion.

Referee #3:

The ms of Malerod et al focuses on the cellular and molecular analysis of the function of ALIX/PDCD6IP in spindle positioning. The authors use a synopsis of experiments from Drosophila (wt/mutant flies) and human cells to show hitherto unknown ALIX functions. They come up with the conclusion that ALIX both helps to nucleate (in parts via -gTuRC) and to stabilise (via MAP1S) spindle microtubules. These functions are generally required to maintain robust astral MT stability and spindle positioning. The data is mostly intriguing, novel and potentially interesting for a broad readership. However, I see a number of issues to be experimentally and formally addressed before the paper could be considered for publication in EMBO J.

Major concerns:

1. The functional experiments in Drosophila describing the role of ALIX in spindle positioning are of excellent quality and comprehensive (different cell types, quantification, verified (at least in parts) by rescue experiments. Likewise, a characterisation is shown of the role of PDCD6IP in human cells and organotypic cell culture that underlines its role in spindle orientation/positioning. However, the knockdown conditions are not verified by applying any rescue experiment; at least different siRNA conditions may be used to show a correlation between severity/penetrance of phenotypes and knockdown efficiency. Moreover: what are the consequences of the ALIX knockdown when going through mitosis: are timing, chromosome segregation fidelity, mitotic exit affected? What about mitotic indices, proliferation rates?

2. Mass spectrometry identification of ALIX interaction partners is certainly an interesting information on its own. However, no details on the ALIX-interactome are provided. Which are the apparently specific interaction partners? Why was MAP1S picked for further analysis? What about g-TuRC components? How does the observed interaction pattern match with interactions patterns from databases? https://thebiogrid.org sees g-tubulin but neither MAP1S nor GCPs in human cells. Due to my opinion, the mass spectrometry data set should be shown, including a validation of major interaction partners that have been identified (at least MAP1S and g-TuRC components) to reinforce the reliability of predicted interactions.

3. Experiments describing the functional and molecular interaction between g-TuRC and and ALIX remain obscure. Saying that ALIX regulates gTuRC stability is at least misleading: equally (slightly) reduced level of g-tub and dGrip91 in ALIX mutant flies (why not showing GCP localisation in HeLa cells after ALIX kd?) suggest mislocalisation of the complex as a whole but not separation of subunits/complex destabilisation. How can we interpret the interaction with g-tub and D Grip91?
alone? No biochemical evidence supports the interaction with the complex (sucrose gradients, Co-IP from whole cell lysates, mass spectrometry: see above). Some mislocalisation of g-TuRC can be observed upon reducing ALIX levels, along with reduced nucleation. However, the functional (indirect) assay tells us that spindle orientation defects are stronger in ALIX mutants than in g-tubulin C ko flies. A nucleation assay under these conditions may help to clarify this issue. Concerning the interaction between MAP1S and ALIX: Apparently, these two proteins do not influence each other in spindle localisation although interacting with, and functionally affecting each other, right? The aspect of a possible interdependent localisation is, however, not clearly addressed. This should be clarified.

Further concerns:

ALIX centrosomal localisation... is certainly clear, including some sort of asymmetry in Prometaphase; however, ALIX seems to be more asymmetric than Asl both in Prometaphase and in Anaphase (see also white colour in overlay). This should be sorted out. In mitotic HeLa cells, ALIX may also have a pool that localises to centriolar satellites, which may be addressed using a colocalisation with centriolar satellite markers (interphase could be easier to analyse as mitosis only). Along these lines: could there be relocalisation from the PCM to satellites upon loss of Cep192 and Cep215 (see Fig. 2E)?

Figure 2: pulldown assay: I am not sure how to interpret the double band of spd2 that comes down with GST ALIX, it certainly does not look like the input lane.

In Figs 5A and B, the way the intensity of astral MTs is quantified remains unclear. I could neither find further specifications in Fig. legends nor in methods. Images in Fig. 5A suggest a stark difference at single cell level. Are other cells not affected at all?

In Fig. 5 C and D, we see slower regrowth kinetics but also still fewer MT in the last time point. Do the mutant/knockdown cells ever catch up at later time points? Experiments in Figure 7 indicate that some difference in total MT fluorescence remains but this difference is by far not as dramatic as in the final time point after regrowth. This should be clarified.

Minor issues:

A centriolar marker may help to unambiguously localise centrosomes after Cep192/Cep215 kd.

Figure 3A, 4A, 4D: indications of significance should be integrated into the graphs.

Throughout all figures and result description, the authors often indicate a "tubulin" signal although, in fact, the fluorescence intensity of microtubules has been determined. Figure marks, legends and results should say "microtubules" instead of "tubulin" where appropriate.

The blot in Fig. 6B is hard to interpret without quantification, which should be done at this point to underline to cooperative functions of these two factors.

Figure 8E displays strong/obvious spindle staining of YFP alone ("empty" YFP sounds strange to me) in control and ALIX kd cells, why is that?

Point-by-point response to the referees’ comments for EMBOJ-2017-97741R - Centrosomal ALIX regulates mitotic spindle orientation by modulating astral microtubule dynamics

All responses to the Referee comments below, as well as all changes made to the text in the revised version of the manuscript, are highlighted with blue text.
Referee #1:

In this manuscript, Malerod et al. describe the first characterization of the function of centrosomal ALIX, a protein known to regulate cytokinesis, endosomal biogenesis, virus release and plasma membrane repair in an ESCRT-dependent manner. Previous studies (including one by the authors) already reported that a pool of ALIX is present at the centrosomes in interphase and during cell division. However, the potential role of centrosomal ALIX during cell division was not known. Using a variety of models (neuroblast divisions, germ cell divisions, epithelial cell divisions in Drosophila as well as human Caco2 organoids and HeLa cells), the authors show that ALIX promotes correct spindle orientation by regulating the nucleation of astral microtubules and their stability. The first regulation depends on the recruitment of the MT nucleator gamma-TURC to centrosomes and the second regulation on the recruitment of the MT binding protein MAP1S. Finally, the authors demonstrate that, both in Drosophila and in Human cells, Cep192 recruits ALIX to the PCM.

Overall, this is an excellent study with careful analysis and important conclusions that deserves to be published in the EMBO Journal. The use of several models clearly backs up the conclusion that ALIX contributes to astral microtubule nucleation/stability and thus to spindle orientation. I have nevertheless a few questions that should be addressed before recommending publication.

Major points

1- All the presented data converge towards a role of ALIX at the centrosome in spindle orientation. However, ALIX has many other functions outside the centrosome. For instance, ALIX could regulate endosomal trafficking, which could indirectly control spindle orientation. It would be therefore important to deplete ESCRT components (e.g. CHMP4B) and demonstrate that there is no spindle orientation defects in these conditions. The Reviewer does not ask to test this in all presented models, but in the most convenient one (e.g. Caco2 organoids or HeLa cells in culture).

• We have addressed this interesting and important issue by determining the mitotic spindle orientation in HeLa cells depleted of ALIX, Hrs (ESCRT-0) or CHMP4B (ESCRT-III) (new Figure EV2F, G). All the proteins are efficiently knocked down (verified by Western blotting analysis, new Figure EV2G), but only ALIX-depleted cells show a spindle misorientation compared to control cells (p<0.001, ANOVA), suggesting that ALIX influences spindle orientation independently of the ESCRT machinery.

• These new Results are described on page 12 (upper paragraph) and in the corresponding Figure legend page 44.

2- The part regarding MAP1S is the only section of the manuscript which is not entirely convincing. In particular the conclusion based on Fig. 7E that ALIX regulates the recruitment of MAP1S is not really significant (74% vs. 84% with no quantification of the MAP1S levels upon ALIX depletion). In addition, results from Figure 1F are interesting but, in my opinion, do not support the fact that ALIX recruits MAP1S but rather that MAP1S can stabilize MTs independently from ALIX. Indeed, if ALIX recruited MAP1S, why MAP1S could rescue the instability of MTs in the absence of ALIX? The results instead argue for two independent pathways.

I therefore suggest the two following experiments to backup this part of the manuscript:

1) Does the overexpression of any MAPs rescue the defects in astral MT length after ALIX depletion? If it were the case, the authors should revise the conclusion of the MAP1S section.

• In order to address the raised comment, we obtained a pEGFP-CLASP2a plasmid from Professor Anna Akhmanova. CLASP2 is known to stabilize microtubules through cell cortex interactions. HeLa cells transfected with control or ALIX siRNA were re-transfected with pEGFP or pEGFP-CLASP2a the following day, and harvested after 24 hours. As shown in new Figure 8I, ectopic expression of CLASP2a did not affect the length or number of astral MTs in ALIX-depleted cells. In contrast, ectopic expression of MAP1S restored the length of astral MTs to almost the same length as observed in control siRNA+pYFP-cells (new Figure 8G), suggesting that ALIX influences MT stability via MAP1S, but not CLASP2a.
2) Does ALIX affect the endogenous MAP1S staining on centrosomes/spindle? Dallol et al. and Tegha-Dunghu et al. provide nice pictures of endogenous MAP1S. This experiment would more directly answer whether ALIX regulates MAP1S recruitment to the spindle (or specifically to the centrosomes for instance).

- We fully agree that the functional relationship between ALIX and MAP1S was not fully characterized in the original manuscript. In concert with previous studies (by Dallol et al and Tengha-Dunghu et al), we detected endogenous MAP1S at the centrosomes, and this localization was most prominent in interphase cells (see new Figure EV5D), but we also detected in mitotic cells, where it was most prominent in prometaphase (see details below EV5D).

- These new Results are described on page 21 (upper paragraph) and in Figure legends (page 47).

- Already at prometaphase, it seemed that MAP1S spread from the centrosomes and onto the newly forming mitotic spindle (new Figure EV5D), supporting the working hypothesis that MAP1S associates nascent MT filaments as they emanate from the centrosomes and stabilizes them to promote efficient formation of the mitotic spindle. In order to further elucidate whether ALIX affects the centrosomal localization of MAP1S, we assessed the centrosomal accumulation of MAP1S in control and ALIX-depleted HeLa cells. To this end we used interphase cells because of the clear centrosomal staining of MAP1S as described above. As shown in the new Figure 8A, B, significantly less MAP1S was detected at the centrosomes in ALIX-depleted cells as compared to control cells (p<0.05, three independent experiments), suggesting that ALIX impacts centrosomal MAP1S recruitment/stabilization. Importantly, that the centrosomes were not completely devoid of MAP1S suggests that ALIX is one of the proteins recruiting MAP1S there. It is also important to emphasize that MAP1S exhibits MT-binding domains, suggesting that in the absence of ALIX MAP1S most likely can localize to the mitotic spindle, but to a lesser extent, as our data presented in Figures 8A and EV5D indicate. Our new results thus indicate that ALIX recruits/stabilizes MAP1S at centrosomes.

- These new Results are described on page 21 (upper paragraph) and in the corresponding Figure legends (pages 42 and 48).

3- The specificity of the ALIX staining should be demonstrated. Does ALIX signal at the centrosome/PCM disappear in Drosophila Alix1 mutant and in ALIX-depleted HeLa cells?

- We agree that the centrosomal detection of ALIX in both Drosophila and human cells requires further validation. Here we have used two different antibodies against ALIX, detecting Drosophila and human ALIX, respectively. Both antibodies have previously been characterized (Eikenes et al, 2015, PLoS Gen and Cabezas A et al, 2005, J Cell Sci). Specifically, we have previously performed stainings with the antibody recognizing Drosophila ALIX and the pre-immune serum in cultured Drosophila cells, showing a centrosomal staining with anti-ALIX and absence of centrosomal signal with the pre-immune serum (Eikenes et al., 2015, PLoS Gen). To further validate the centrosomal detection of ALIX here, we performed RNAi experiments in NBs and HeLa cells, showing that the centrosomal detection of ALIX was significantly reduced (p<0.05) upon ALIX depletion in both NBs and HeLa cells (see new Figure EV1A, B). The same antibodies were used in the Western blot analyses, showing reduced cellular expression of ALIX compared to control cells. Altogether, these experiments confirm that the antibodies used specifically detect ALIX at the centrosomes in fly and human cells.

- These new Results are described on page 6 (upper paragraph) and in Figure legends on pages 42-43.

Minor points
1- Does ALIX regulate MT nucleation also in interphase cells? The authors could analyze their data for instance in human interphase cells, as shown in Figure 5D.

• We agree that this is an interesting aspect to elucidate and have re-examined immunofluorescence specimens to determine MT regrowth in w1118 and alix1 NBs over time. As shown in the new Figure EV4C, MT-regrowth is significantly delayed in alix mutant interphase NBs, suggesting that ALIX promotes MT nucleation in both dividing and non-dividing cells.

• The new Result is described on page 15 (upper paragraph) and in Figure legends of the Expanded View on page 46.

2- For spindle orientation analysis (N=3 experiments, Figure 3A, 4A, 4D etc.), the authors should provide error bars.

• We have updated all the graphs showing spindle orientation analysis (Figures 3A, 4A, 4D, 6A, 6E, EV1C-D and EV2E-F) with error bars (S.E). We have also included indications of significance in these graphs.

3- It would be interesting to measure the speed of spindle movement after ALIX depletion in at least one model. According to the conclusion of the manuscript, one should expect slow rotation of the spindles in depleted cells.

• We measured rotation speed of the mitotic spindles from centrosome separation until telophase in control and alix3 SOPs in available movies from old Figure 3D, E and new live imaging experiments. All data on the increased spindle rotation angles in alix3 compared to control SOPs are summarized in new Figure 3E. The measurements of rotation speed revealed that is was higher in alix mutant SOPs compared to control cells (p<0.01, see new Figure EV2C). Indeed we reason that this is in accordance with the loss of astral MTs in alix-deprived cells, resulting in less tightly anchored spindles free to rotate faster.

• This new Result is described on page 11 (first two lines) and in Figure legends of the Expanded View page 44.

Referee #2:

In the paper, Malerod and colleagues investigate the role of ALIX at centrosomes in both Drosophila and human cultured cells. They provide evidence that ALIX is recruited to centrosomes via an interaction with Spd-2 and show that ALIX is required for the proper recruitment of g-TuRCs to centrosomes and thus for proper centrosomal microtubule nucleation. They also show that ALIX helps recruit MAP1S to microtubules in HeLa cells. They show that various cell types that lack or have reduced levels of ALIX display mitotic spindle alignment defects, in line with the observed reduction in centrosomal microtubules.

Overall the paper is interesting and will be suitable for publication once the authors have addressed the comments below.

1) The authors use structured illumination microscopy (SIM) to examine the sub-centrosomal localisation of ALIX in Figure 1C, but the images look of poor quality, especially as SIM reconstructions are prone to artefactual meshwork patterns. It would be better to perform line or radial plots on confocal images, which are more reliable and have the resolution required to distinguish the PCM from the centrioles. The authors could plot the fluorescence of a centriole marker (e.g. Asl) and ALIX to show that the ALIX fluorescence is more spread out.

• In the updated manuscript the SIM images are replaced with line scan analysis performed using widefield images (new Figure 1E). In accordance to the widefield images (new Figure 1D), the line scan analysis indicated a broader distribution of ALIX (0.72 µm)
across the centrosome in comparison to the narrower peak of glutamylated tubulin (0.48 µm) enriched at the centrioles (new Figure 1E). The apparently asymmetric distribution of the centriolar marker, skewed to one end of the centrosome, reflects most likely the compressed structure of the centrosome in metaphase cells as previously reported by super resolution imaging (Sonnen et al, 2012, Biol Open). Consequently, we always draw the scan line from the centriolar-positive end of the centrosome, as the arrow indicates in inset 1 of Figure 1D. That ALIX is detected more widely across the centrosome compared to the centriole marker, suggests that ALIX is part of the PCM also in human cells, as seen in NBs.

- The new analysis and Result is described on page 7 (upper paragraph) and in Figure legend (page 35, upper paragraph).

2) In figure 2, the authors quantify the number of centrosomes that lack ALIX signal in different mutant backgrounds, but it would be more accurate and useful to quantify the average fluorescence signal from multiple centrosomes from multiple samples (similar to how they quantified g-tubulin and grip 91 levels in Figure 5E). It is strange that some centrosomes would completely lack ALIX, when ALIX can clearly still be recruited in the absence of either Spd-2 or Cnn. A more realistic representation would therefore be the % drop in ALIX centrosomal levels in the different mutants.

- We fully agree and have calculated the centrosomal intensity of ALIX in the different genotypes. The new graph is presented in new Figure 2B. In summary, ALIX is significantly less detected at the centrosomes in Sas6c0901 NBs (~20% of wild type levels, p<0.05), as expected (new Figure 2B). Furthermore, significantly less ALIX accumulates at the centrosomes in DSpd-2hk214d and cnnhk21 NBs (~30% of wild type levels, p<0.05 and p<0.01, respectively), suggesting that these proteins facilitate centrosomal recruitment of ALIX (new Figure 2B).
- The new Results are described on page 7 (middle paragraph) and in the corresponding Figure legend on page 35, lower paragraph.
- Accordingly, we have also measured centrosomal intensity of ALIX in HeLa cells depleted of Cep192 or Cep215 and updated the graph in new Figure 2F. The centrosomal intensity of ALIX is decreased to ~30% (p<0.01) and ~40% (p<0.01) upon Cep192 and Cep215 depletion, respectively.
- The new Results are described on page 8 (upper paragraph) and in Figure legends (page 36, upper paragraph).

3) The authors conclude that ALIX is recruited to centrosomes via a direct interaction with Spd-2, but ALIX can still be recruited in the absence of Spd-2. Thus, although Spd-2 may be the major recruiter of ALIX, ALIX may also be recruited by other means - the authors should discuss this.

- Indeed this is an important comment, and we agree that DSpd-2 most likely does not represent the only recruiter of ALIX to the centrosomes. Therefore, in the updated version of the Discussion of the revised manuscript we have clarified our view on page 23, upper paragraph.

4) I would recommend that the authors quantify centrosomal asters in alix clones during prophase. It is very difficult to quantify centrosomal asters in Drosophila neuroblasts during metaphase because the spindle fluorescence is very dominant, even in WT neuroblasts (as can be seen in Figure 5). It is much better to quantify asters during prophase, before the spindle has formed. To do this, they just need to stain for centrioles (Asl) and mitotic DNA (Phospho-histone H3).

- We understand the points raised by the reviewer completely. To elucidate the molecular mechanism explaining how ALIX influences mitotic spindle orientation, we have focused calculating the percentage of astral MTs in metaphase cells, since these filaments facilitate the anchoring of the spindle to the cell cortex. In the revised manuscript we have now investigated delayed MT regrowth in interphase cells (see the new Figure EV4C), and it is tempting to speculate that we would most likely obtain similarly delayed MT regrowth when examining prophase cells.

5) In Figure EV4A, the asters in the bottom images do not look different between the mutant and the control. Rather, it seems that the control just has a higher contrast.
We agree that the background around the control cell seems to have higher contrast. However, careful examination of the images revealed that this apparently high background is caused by the presence of intercellular bridges of cytokinetic, neighbouring cells. In order to clarify the reduction of astral MTs in the alix<sup>-/-</sup> SOP clones, we have presented zoom-in images of the spindle poles and highlighted the presence of astral MTs in control cells with arrows (see **new Figure EV4A**).

In the revised manuscript, the Expanded View Figure legend is correspondingly updated (page 46, first line).

6) To assess g-tubulin and Grip91 levels at centrosomes in Figure 5E, the authors need to stain with a centriole marker, such as Asl, to be sure that the g-tubulin signal is coming from a centrosome.

We agree that including a centrosomal marker is required to confirm that the γ-tubulin and Dgrip91 signals actually come from the centrosomes. We have in the updated figure panels included all the original staining. Specifically, Cnn marks the centrosomes in con and alix<sup>-/-</sup>-FRT82B NBs stained with γ-tubulin (**new Figure 5E**) and γ-tubulin stained the mitotic spindle, thus indicating the spindle poles, in con and alix<sup>-/-</sup>-FRT82B NBs where the centrosomal localization of Dgrip91 is examined (**new Figure 5E**). Importantly, our new analysis also indicates that in difference to the reduced accumulation of γ-tubulin and Dgrip91 in alix-deprived NBs, the centrosomal localization of Cnn remains unaffected (**new Figure 5E**).

The corresponding Figure legend is updated on page 38, lower paragraph.

7) I have major concerns with the conclusions from the double mutant and double knockdown experiments in Figure 6. The authors use an increase in phenotype in the double mutants/knockdowns to conclude that ALIX also functions independently of g-tubulin. This can only be concluded where the mutants/knockdowns result in a complete absence of protein (e.g. a null mutant). In the blot in Figure 6B, there is still a faint band of g-tubulin, and in our lab we have found that when we immunostain g-tubulin null mutant 3rd instar larval brains for g-tubulin, we can detect g-tubulin at centrosomes in many of the cells. We therefore believe that the maternal contribution of g-tubulin shows strong perdurance, and this fits with the weak phenotypes observed by these authors, ourselves and others. The presence of protein is also a problem in the RNAi knockdowns in human cells, where the blot in Figure 6D shows that there is plenty of protein left. The increased phenotypes seen in the double mutants/knockdowns could therefore be due to potentiation of the ALIX-g-tubulin pathway i.e. the g-tubulin mutant/knockdown reduces, but does not abolish, g-tubulin from the centrosome, and adding in the ALIX mutant/knockdown just makes this worse. I therefore think that the authors need to tone down their conclusions, explaining their data is consistent with a model of separate pathways, but it is not conclusive.

We fully agree with the comments raised by the Referee. We appreciate the shared information likely explaining the weak phenotype observed in γTub23C<sup>PI</sup>-NBs and γ-tubulin -depleted HeLa cells. In order to further characterize the functional relationship between ALIX and g-tubulin, we have included new analyses in the revised manuscript:

- First, the expression levels of ALIX and γ-tubulin in the single and double mutant NBs or single and double depleted HeLa cells were quantified (shown below the corresponding Western blots in **new Figure 6B and F** respectively). Interestingly, we did not observe any differences in the remaining expression of both ALIX and γ-tubulin in the single versus double mutants/knockdown cells.
- Second, we performed a MT regrowth assay in w<sup>1118</sup>, alix<sup>-/-</sup>, γTub23C<sup>PI</sup> and γTub23C<sup>PI</sup>; alix<sup>-/-</sup> NBs illustrating that the two single mutant NBs possessed the same low MT nucleation capacity as the double mutant NBs (see **new Figure 6C, D**). Interestingly, this experiment supports rest of our findings that ALIX influences MT nucleation via γ-tubulin, since loss of ALIX did not reduce the low MT regrowth further in γTub23C<sup>PI</sup> NBs. Furthermore, that ALIX worsens the spindle misorientation defect in γTub23C<sup>PI</sup> cells, indicates that ALIX influences spindle orientation by also an additional molecular mechanism to γ-tubulin.

These new experiments and Results, along with a discussion about the points raised by the referee, are described on page 17 and in the updated Figure legends (page 39 (lower paragraph) to 40 (upper paragraph)).
8) I am unsure how spindle orientation can be measured after microtubule depolymerisation in Figure 7?

- Due to lack of space we did not show the confocal micrographs corresponding to the graph presented in Figure 7A and B of the original manuscript. We stained all the samples with a centrosome marker, γ-tubulin, to be able to indicate the spindle axis even in specimens where the mitotic spindle was depolymerised due to incubation on ice. Images from the FEC experiments in Figure 7A are shown here below. We have also included an explanation of this point for these experiments in the Figure legends (page 40, lower paragraph).

9) The conclusion that "ALIX controls microtubule stability" (p.17) is too strong. "Influences" would be more accurate.

- We agree, and have moderated our statement accordingly. See the revised manuscript page 19, upper paragraph.

10) The authors need to present their mass spec data

- In the revised manuscript we present the full mass spectrometry dataset of proteins detected in the MT-pellet of control and ALIX-depleted HeLa cells in Dataset EV1.
- We have also highlighted MAPs, GCPs and γ-tubulin detected in the polymerized MT-pellet of control and ALIX-depleted HeLa cells detected in the mass spectrometry analysis in new Figure EV5A. Furthermore, we have validated three more candidates, in addition from MAP1S, of the detected proteins, namely CLASP2, GCP3 and γ-tubulin (see new Figure EV5B).
- These new Results are described on page 19-20 and in the Expanded View Figure legends (page 46, lower paragraph) in the revised manuscript. The mass spectrometry analysis is also described in the Expanded View Materials and Methods section of the revised manuscript (pages 9 (lower paragraph) and10 (upper paragraph).

11) Does the relatively small change in MAP1S association with microtubules in the absence of ALIX (Figure 7E) correspond to the differences seen in the mass spec?

- MAP1S was only detected in the MT-pellet fraction of control cells and not ALIX depleted cells (new Figure EV5A and Dataset EV1). Our Western blot analysis confirms that significantly less MAP1S associates MT-filaments upon ALIX knock down (p<0.05, new Figure EV5B). It is important to emphasize the semi-quantitative aspect of mass spectrometry analysis, implying that the Mascot score between two samples must be at least 2.5 fold different to represent a true difference. Concerning γ-tubulin, which was less than 2.5 fold accumulating in ALIX-depleted cells, its levels were not confirmed to be significantly changed by Western blotting analysis, illustrating that mass spectrometry analysis are much less quantitative than for instance Western blotting analysis, and further demonstrates the importance of validating mass spectrometry candidates.
12) The IPs in Figure 7 were apparently performed under conditions that would keep MTs in the extracts, so any perceived interaction could be due to both MAP1S and ALIX being associated with microtubules. If so, the difference in association between ALIX and different MAPS1 constructs may reflect the difference in the ability of the MAPS1 constructs to bind microtubules.

- It is true that the GFP-trap IP was performed in cells with intact MTs. In light of the new results presented in new Figure 8A, B, suggesting that ALIX is important for recruiting MAP1S to the centrosomes (see also response to Referee #1, Major point 2-2), we hypothesize that ALIX and MAP1S are in the same biological complex regardless of the intact MT filaments. Furthermore, MAP1S-LC interacts with MTs, but does not interact strongly with ALIX, whereas the MAP1S-HC more efficiently brings down ALIX in the co-IP in new Figure EV5C. These results indicate that the contribution of MTs to the association between ALIX and MAP1S might not be major in this experiment.

Minor comments:

a) The authors refer to sequential recruitment of the PCM in their introduction (p.3), and they conclude in their results that ALIX is recruited "relatively early during PCM assembly" (p.7). It is worth noting, however, that the PCM is not assembled sequentially. Instead, PCM components build up together during centrosome maturation i.e. if component A recruits component B, they still build up in levels together; component A does not fully build up before component B. The authors should therefore adjust their wording and conclusion.

- We have rewritten our description of PCM in the Introduction (page 3) and rephrased the conclusion about ALIX recruitment in the Results section (page 7).

Referee #3:

The ms of Malerod et al focuses on the cellular and molecular analysis of the function of ALIX/PDCD6IP in spindle positioning. The authors use a synopsis of experiments from Drosophila (wt/mutant flies) and human cells to show hitherto unknown ALIX functions. They come up with the conclusion that ALIX both helps to nucleate (in parts via -gTuRC) and to stabilise (via MAP1S) spindle microtubules. These functions are generally required to maintain robust astral MT stability and spindle positioning. The data is mostly intriguing, novel and potentially interesting for a broad readership. However, I see a number of issues to be experimentally and formally addressed before the paper could be considered for publication in EMBO J.

Major concerns:

1. The functional experiments in Drosophila describing the role of ALIX in spindle positioning are of excellent quality and comprehensive (different cell types, quantification, verified (at least in parts) by rescue experiments. Likewise, a characterisation is shown of the role of PDCD6IP in human cells and organotypic cell culture that underlines its role in spindle orientation/positioning. However, the knockdown conditions are not verified by applying any rescue experiment; at least different siRNA conditions may be used to show a correlation between severity/penetrance of phenotypes and knockdown efficiency. Moreover: what are the consequences of the ALIX knockdown when going through mitosis: are timing, chromosome segregation fidelity, mitotic exit affected? What about mitotic indices, proliferation rates?

- We fully agree that phenotypes observed in knockdown experiments should be validated. We have used three different siRNA oligoes targeting ALIX in our analyses of mitotic spindle orientation in Caco-2 cysts, which all show that lack of ALIX induces a spindle misorientation phenotype as shown in Figure 4D and new Figure EV2E as described in the revised manuscript. Specifically, for all three ALIX siRNAs used ALIX-depleted cells showed a significantly greater variation in mitotic spindle misorientation compared to control cells (p<0.001, Figure 4D and p<0.05, Figure EV2E). We have verified the
efficient ALIX depletion with all three siRNAs by Western blot analysis (Figure 4E and Figure EV2D). The ALIX siRNAs used were 1) ALIX siRNA (Figure 4D), 2) ALIX siRNA #2 and 3) ALIX siRNA #3 (Figure EV2E) and are specified in the Expanded View Materials and Methods.

- Corresponding the Results (page 11, lower paragraph) and Expanded View Figure legends (page 44, upper paragraph) are updated.
- Characterizing the overall effect ALIX depletion exerts on cell cycle progression is very interesting. In the current manuscript we have performed this by live cell imaging of HeLaK cells depleted or not of ALIX (new Figure EV21). We have timed mitosis progression in these cells and have now in addition to the length of prometaphase and metaphase as shown before, in the revised manuscript also measured the duration of anaphase, telophase and cytokinesis as shown in new Figure EV21. In comparison to control siRNA cells, ALIX deprived cells take longer to complete prometaphase (21.6±1.4 minutes) and metaphase (73.6±14.8 minutes), the latter phenotype is often observed in cells with spindle misorientation, compared to control cells (16.8±0.9 and 39±4.2 minutes, respectively) (p<0.05). Interestingly, ALIX-depleted cells tend to be arrested in metaphase (9.1%), meaning that they did not progress to anaphase during the 18 hours of imaging. A significant proportion of these cells gave up metaphase and died (mitotic exit) (68.2% compared to 2.3% in control cells) (p<0.001) (Figure EV2J). Furthermore, in concert with previous studies (Carlton J et al, 2007, Science and Morita E et al, 2007, EMBO J), ALIX-depleted cells showed a delay in cytokinesis (60.4±10.7 minutes) compared to control cells (41.0±2.4 minutes) (p<0.05) (Figure EV21). Altogether, elucidating the effect ALIX depletion exerts on cell proliferation deserves an even more careful investigation, in which the cells are followed through subsequent cell cycles by live cell imaging. We would like to address these and the other issues raised by the referee further in both cultured cells and Drosophila models in the future, but lack of time as well as microscopes that allow imaging several cell cycle progresses gently enough are at present limiting factors.

2. Mass spectrometry identification of ALIX interaction partners is certainly an interesting information on its own. However, no details on the ALIX-interactome are provided. Which are the apparently specific interaction partners? Why was MAP1S picked for further analysis? What about g-TuRC components? How does the observed interaction pattern match with interactions patterns from databases? https://thebiogrid.org sees g-tubulin but neither MAP1S nor GCPs in human cells. Due to my opinion, the mass spectrometry data set should be shown, including a validation of major interaction partners that have been identified (at least MAP1S and g-TuRC components) to reinforce the reliability of predicted interactions.

- We agree and in the revised manuscript we present the full mass spectrometry dataset of proteins detected in the MT-pellets of control and ALIX-depleted HeLa cells in Dataset EV1.
- In the revised manuscript we also show a table of highlighted mass spectrometry hits of the identified MAPs, GCPs and g-tubulin, in new Figure EV5A. Furthermore, we have validated MAP1S, CLASP2, GCP3 and g-tubulin by Western blot analysis, which showed that indeed MAP1S, CLASP2 and GCP3 are differentially detected in the MT-pellet fractions isolated from ALIX-depleted cells compared to control cells (new Figure EV5B).
- In our MS analysis, MAP1S was identified in MT-enriched fractions of both mock and control siRNA-transfected cells, but not in ALIX KD cells (new Figure EV5A and Dataset EV1) and our Western blotting analysis confirms that significantly less MAP1S associates MT-filaments upon ALIX knock down (p<0.05, new Figure EV5B). MAP1S was selected for further analyses because it has previously been shown to regulate MT stability in dividing cells (Dallol et al, 2007; Halpain & Dehmelt, 2006; Orban-Nemeth et al, 2005; Tegha-Dunghu et al, 2014).
- To our knowledge, the interactome of MT-pellet fractions of control and ALIX-depleted cells has not been characterized before, and therefore it is hard to compare our mass spectrometry results with results presented in ALIX interactome databases. Thus, the results obtained in our current study indicate proteins putatively accumulating in the polymerized MT-pellet fractions in an ALIX-dependent manner, and therefore cannot directly be compared to previous results characterizing the ALIX-interactome.
These new Results are described on pages 19-20 and in the Expanded View Figure legends (pages 46-47) in the revised manuscript, along with explanations of why MAP1S was picked for further analysis. The mass spectrometry analysis is also described in the Expanded View Materials and Methods section of the revised manuscript (pages 9-10).

Please also see responses to Referee #2, Major comment 10).

3. Experiments describing the functional and molecular interaction between g-TuRC and and ALIX remain obscure. Saying that ALIX regulates gTuRC stability is at least misleading: equally (slightly) reduced level of g-tub and Dgrip91 in ALIX mutant flies (why not showing GCP localisation in HeLa cells after ALIX kd?) suggest mislocalisation of the complex as a whole but not separation of subunits/complex destabilisation. How can we interpret the interaction with g-tub and Dgrip91 alone? No biochemical evidence supports the interaction with the complex (sucrose gradients, Co-IP from whole cell lysates, mass spectrometry: see above). Some mislocalisation of g-TuRC can be observed upon reducing ALIX levels, along with reduced nucleation. However, the functional (indirect) assay tells us that spindle orientation defects are stronger in ALIX mutants than in g-tubulin C ko flies. A nucleation assay under these conditions may help to clarify this issue.

- We agree that elucidating the functional relationship between γ-tubulin and ALIX in the regulation of spindle orientation is challenging. In the revised manuscript we show mass spectrometry data indicating that GCP3 and γ-tubulin are differently associated the polymerized MT-pellet fraction in ALIX depleted HeLa cells (new Figure 5A). However, only GCP3 was validated to less abundantly associate with the MT-pellet fraction by Western blot analysis (new Figure EV5B). The discrepancies between mass spectrometry and Western blot analysis illustrate the semi-quantitative aspects of the first method and emphasize the importance of validating results by alternative methods. However, we don’t think that these aforementioned results (Figure EV5A, B) conflict with our observations of reduced centrosomal accumulation of γ-tubulin and Dgrip91 upon ALIX depletion (Figure 5E, H), since the MT-pellet fractions contains MT filaments as well as centrosomes.

- In addition to the in vitro binding assay, we also present data in the revised manuscript showing that GST-ALIX, but not GST, pulls out both γ-tubulin and Dgrip91 from larval brain lysates (new Figure 5F), indicating that these three proteins are in the same biological complexes.

This new Results is described on page 15 (lower panel), Figure legends (page 39, upper paragraph) and in the Material and method section (Expanded View page 8).

Interestingly, when determining the MT nucleation capacity in w 1118, alix+, γTub23CΔ1 and γTub23CΔ2; alix+ NBs, we observed the same delayed MT regrowth in single mutants as in double mutants, illustrating that the two single mutant NBs possessed the same low MT nucleation capacity as the double mutant NBs (see new Figure 6C, D). This experiment supports rest of our findings that ALIX influences MT nucleation via γ-tubulin, since loss of ALIX did not reduce the low MT regrowth further in γTub23CΔ2 NBs. And furthermore, that ALIX worsens the spindle misorientation defect in γTub23CΔ2 cells, indicates that ALIX influences spindle orientation by also an additional molecular mechanism to γ-tubulin.

- These new Results are described on page 17 (upper paragraph) and in Figure legends (page 40, upper paragraph).

Concerning the interaction between MAP1S and ALIX: Apparently, these two proteins do not influence each other in spindle localisation although interacting with, and functionally affecting each other, right? The aspect of a possible interdependent localisation is, however, not clearly addressed. This should be clarified.

- Indeed we agree that the functional relationship between ALIX and MAP1S was not fully characterized in the original manuscript. However, we now present data indicating that ALIX recruits MAP1S to the centrosomes (new Figure 8A, B). Specifically, we detected endogenous MAP1S at the centrosomes, and this localization was most prominent in interphase cells (see new Figure EV5D), in concert with previous studies (by Dallol et al), but we also detected in mitotic cells, where it was most prominent in prometaphase (see details below and Figures 8A and EV5D). To further elucidate whether ALIX affects the centrosomal localization of MAP1S, we assessed the centrosomal accumulation of MAP1S in control and ALIX-depleted HeLa cells. To this end we used interphase cells because of
the clear centrosomal staining of MAP1S as described above. As shown in the new Figure 8A, B, significantly less MAP1S was detected at the centrosomes in ALIX-depleted cells as compared to control cells (p<0.05, three independent experiments), suggesting that ALIX impacts centrosomal MAP1S recruitment. Importantly, that the centrosomes were not completely devoid of MAP1S suggests that ALIX is one of the proteins recruiting MAP1S there. Our new results thus indicate that ALIX recruits MAP1S to centrosomes.

- This new Results is described on page 21 (upper paragraph) and in Figure legends (page 41).

Further concerns:

ALIX centrosomal localisation... is certainly clear, including some sort of asymmetry in Prometaphase; however, ALIX seems to be more asymmetric than Asl both in Prometaphase and in Anaphase (see also white colour in overlay). This should be sorted out.

- The apparently asymmetric distribution of ALIX between the two centrosomes was further investigated. Our new data show that indeed the intensity of ALIX, as well as of another PCM protein, Cnn, was 2-fold higher at the strongest compared to the weakest centrosome (new Figure 1B). In contrast, the centriolar marker Asl did not show the same asymmetry (ratio = 1.2). These data may indicate that an asymmetry of the PCM amount remains to some extent at least during metaphase.
- The new Result is described on page 6 (lower paragraph) and in Figure legends (page 1, upper paragraph).

In mitotic HeLa cells, ALIX may also have a pool that localises to centriolar satellites, which may be addressed using a colocalisation with centriolar satellite markers (interphase could be easier to analyse as mitosis only). Along these lines: could there be relocalisation from the PCM to satellites upon loss of Cep192 and Cep215 (see Fig. 2E)?

- Indeed we find this comment very interesting, and tried to address this by ectopically expressing CSPP-L, known to localize to centriolar satellites (Patzke S et al, 2006, J Cell Physiol), in HeLa cells and assess the putative co-localization with ALIX. Our preliminary results did not show convincing co-localization between the two proteins, but we also feel that this question deserves a more thorough investigation in the future.

Figure 2: pulldown assay: I am not sure how to interpret the double band of spd2 that comes down with GST ALIX, it certainly does not look like the input lane.

- In vitro translation of protein larger than 100 kDa is generally difficult and gives low yield. Unfortunately, an air bubble at the right edge of the input lane masks the band and makes it hard to spot the double band also in the input lane in Figure 2C. This double band is clearer in the GST-ALIX-lane due to less background, probably caused by protein degradation.

In Figs 5A and B, the way the intensity of astral MTs is quantified remains unclear. I could neither find further specifications in Fig. legends nor in methods. Images in Fig. 5A suggest a stark difference at single cell level. Are other cells not affected at all?

- In the revised manuscript we have included a separate description of how the intensity of astral MTs is calculated (page 26, lowest paragraph). In accordance to the point raised by referee #2 (major point 4, page 5 of this Commentary), the astral MTs in metaphase NBs are much weaker than the spindle MTs, which may give some variations in the quantifications, but overall most of ALIX-deprived cells display less astral MTs as shown in Figure 5.

In Fig. 5 C and D, we see slower regrowth kinetics but also still fewer MT in the last time point. Do the mutant/knockdown cells ever catch up at later time points? Experiments in Figure 7 indicate that some difference in total MT fluorescence remains but this difference is by far not as dramatic as in the final time point after regrowth. This should be clarified.
We have performed MT regrowth analysis up to 30 minutes after cold-induced MT-depolymerization in interphase NBs, and still observe reduced MT intensities in alix<sup>−/−</sup> NBs compared to w<sup>118</sup>. These data are thus in accordance with our observations showing less total MTs in alix-deprived cells compared to control cells.

Minor issues:

A centriolar marker may help to unambiguously localise centrosomes after Cep192/Cep215 kd.

- In the original confocal micrographs the cells were also immunostained with anti-a-tubulin indicating the mitotic spindle with the spindle poles in each end. Therefore, in new Figure 2E, the a-tubulin-staining is also included to highlight the position of the centrosomes in the cells. The corresponding Figure legends is updated, see page 36, upper paragraph.

Figure 3A, 4A, 4D: indications of significance should be integrated into the graphs.

- We have updated all the spindle orientation analyses (Figure 3A, 4A, 4D, 6A, 6E, EV1C-D and EV2E-F) with indications of significance in the graphs, as well as with error bars (S.E).

Throughout all figures and result description, the authors often indicate a "tubulin" signal although, in fact, the fluorescence intensity of microtubules has been determined. Figure marks, legends and results should say "microtubules" instead of "tubulin" where appropriate.

- We agree that when the MT intensity is measured this should be indicated and we have updated the relevant graphs in Figures 5, 6C, 7C-D, 8C-E, 8G-H, EV4A-C. However, tubulin is kept on figure marks since these indicate that an anti-a-tubulin antibody was used.

The blot in Fig. 6B is hard to interpret without quantification, which should be done at this point to underline to cooperative functions of these two factors.

- The results of quantifications of independent Western blots are presented in new Figures 6B and 6F in order to determine the protein levels of ALIX and γ-tubulin in single and double mutants and knockdown cells. Importantly, the quantifications show similar extent of depletion in both single and double mutants/knockdowns. We discuss these results further on pages 17-18 in the revised manuscript.

Figure 8E displays strong/obvious spindle staining of YFP alone ("empty" YFP sounds strange to me) in control and ALIX kd cells, why is that?

- GFP and YFP are often massively expressed in cells, and since the proteins appear to be sticky they also seem to decorate the mitotic spindle in cells with very high expression.

Finally, we would like to thank all the Referees for their valuable and constructive comments that have contributed to greatly improving our manuscript.

2nd Editorial Decision 22 January 2018

Thank you for submitting your revised manuscript for our consideration. We have now received the re-reviews from all three original referees, and I am pleased to inform you that they all consider the manuscript significantly improved and therefore in principle suitable for EMBO Journal publication. Nevertheless, referee 3 still retains several specific reservations, which I feel need to be clarified/addressed prior to ultimate acceptance. I am therefore returning the manuscript to you once more for a final round of (minor) revision, to allow you to answer to these remaining points through further explanations and/or additional supporting data and controls.

Once we will have received the re-revised manuscript files addressing the remaining editorial and
referee points, we should hopefully be in a position to proceed with eventual acceptance and publication of the study. Please do not hesitate to get back to me should you have any further questions in this regard.

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REFEREE REPORTS

Referee #1:

The authors convincingly answered my questions in the revised manuscript. I therefore recommend publication.

Referee #2:

The authors have worked hard to address nearly all of the reviewers concerns. They have adjusted some conclusions according to the reviews and added new data that supports their model. I therefore believe the paper is ready for publication in EMBO.

Referee #3:

Malerod et al. now hand in a revised version of their ms on the function of ALIX on spindle orientation and astral MT dynamics. I do appreciate the authors' efforts to adequately address all reviewers concerns, which were versatile and rich in detail. Their study represents an amazing integration of in vivo approaches in the fly with experiments in human cell culture, which should be communicated to a broad readership.

In terms of the major concerns I had raised in my review, I am still left with mixed feelings on some of the data. To my opinion, the issues listed below have to be fixed to qualify the manuscript for publication in EMBO J.

The authors back up ALIX knockdown with alternative conditions (2 new siRNAs), which give very consistent results. Small detail: Fig. EV2, panel D tells us that there were no cells/spindles at the angles between 60 and 75° in all independent/separate experiments, is this correct? The overall samples size is around 40; are these all the structures counted or does this reflect one of three experiments? If so, the sample size is rather small.

I appreciate live imaging in human cells after ALIX kd to reveal potential mitotic abnormalities here. Delays in different mitotic stages are quantified, however, also on the basis of a rather small sample size, i.e. 44 structures in all experiments (?) together (meaning ca 15 in each one of three experiments). Was it also done with just one siRNA? If these data are shown (yes, I think they should), they ought to be more robust.

Functional interaction between ALIX and MAP1S as well as γ-TuRC was evaluated by microtubule sedimentation experiments in the presence or absence (kd) of ALIX in human cells. The corresponding full data set is now shown. The suppl. table lists many proteins based of the identification of one "significant" sequence without telling us about the sample origin (control or knockdown?). I still very much like the approach and I also understand that a microtubule sediment contains many more proteins than alpha tubulin and MAPs, but the way they data are presented (no legend that explains the categories, no clear assignment of ratios between control and knockdown, no information about replicates (just one experiment?); it would be good to label those proteins in the large table, which are shown in the small table in EV5A) does not fully support the conclusions drawn. Likewise, the new Western blot data (Fig. EV5B) neither show dramatic changes in the representation of MAP1S nor γ-TuRC on microtubules after ALIX knockdown.

Interaction of γ-TuRC with ALIX: the new pulldown experiment in Fig. 5F suggests interaction between the complex and ALIX: fine. A negative control, i.e. detection of any protein that does neither bind to GST nor to GST-ALIX, would strengthen this important result.
The authors now show a reduction of the centrosomal MAP1S signal after ALIX knockdown. This data would be stronger if a centrosomal marker was shown that was not altered and may have served as an internal control for centrosome position and quantification of the PCM and/or centrioles in general. The same applies to Fig. 5H showing a reduction in centrosomal γ-tub signals after ALIX knockdown.

The authors insist that GFP/YFP alone accumulate on spindles in an unspecific manner when overexpressed. How can we then interpret spindle localisation of MAP1S or CLASP in then same panel? Just less protein expressed and then specificity regained? I find this hard to believe.

Minor issues:
p. 16 top: Correspondingly ALIX depleted cells showed only 62...
p. 45, fig. legend EV2: apoptosis as a consequence of metaphase arrest is not commonly refereed to as "mitotic exit".

2nd Revision - authors’ response 8 April 2018

Point-by-point response to the referees’ comments for EMBOJ-2017-97741R1 - Centrosomal ALIX regulates mitotic spindle orientation by modulating astral microtubule dynamics

All responses to the Referee comments below are highlighted with blue text. All changes made to the text in the re-revised version of the manuscript are indicated in blue text.

Referee #1:

The authors convincingly answered my questions in the revised manuscript. I therefore recommend publication.

Referee #2:

The authors have worked hard to address nearly all of the reviewers concerns. They have adjusted some conclusions according to the reviews and added new data that supports their model. I therefore believe the paper is ready for publication in EMBO.

Referee #3:

Malerod et al. now hand in a revised version of their ms on the function of ALIX on spindle orientation and astral MT dynamics. I do appreciate the authors' efforts to adequately address all reviewers concerns, which were versatile and rich in detail. Their study represents an amazing integration of in vivo approaches in the fly with experiments in human cell culture, which should be communicated to a broad readership.

In terms of the major concerns I had raised in my review, I am still left with mixed feelings on some of the data. To my opinion, the issues listed below have to be fixed to qualify the manuscript for publication in EMBO J.

The authors back up ALIX knockdown with alternative conditions (2 new siRNAs), which give very consistent results. Small detail: Fig. EV2, panel D tells us that there were no cells/spindles at the angles between 60 and 75{degree sign} in all independent/separate experiments, is this correct? The overall samples size is around 40; are these all the structures counted or does this reflect one of three experiments? If so, the sample size is rather small.

Yes, it is correct that we did not observe any cells with relative spindle orientation between 60 and 75 degrees in Caco-2 cells transfected with ALIX siRNA#3 in these experiments (Fig. EV2E). Despite the relatively small sample size, the misorientation phenotype is significantly different in
ALIX siRNA cells compared to control cells according to the ANOVA statistical test (Fig. EV2E) performed by our collaborating statistician Professor Knut Liestøl. Furthermore, the misorientation phenotype is evident upon ALIX depletion using three alternative siRNA oligoes (Fig. 4D & Fig. EV2E), which collectively strengthens our novel findings that ALIX influences mitotic spindle orientation in human cells.

I appreciate live imaging in human cells after ALIX kd to reveal potential mitotic abnormalities here. Delays in different mitotic stages are quantified, however, also on the basis of a rather small sample size, i.e. 44 structures in all experiments (?) together (meaning ca 15 in each one of three experiments). Was it also done with just one siRNA? If these data are shown (yes, I think they should), they ought to be more robust.

We do agree that it is important to include an adequate number of cells per experiment in order to be able to conclude properly. The sample size refers to the total number of cells in three experiments using one ALIX siRNA oligo (ALIX siRNA#1). Prometaphase, metaphase and cytokinesis were, despite the relatively small cell number, significantly delayed compared to control cells as tested by a Student’s t-test (Fig. EV2I). Importantly, as the table below shows, similar phenotypes, i.e. delayed progression through prometaphase, metaphase as well as cytokinesis compared to control cells is also observed when cells are depleted of ALIX using an alternative siRNA oligo (i.e. ALIX siRNA#2). The experiment summarized in the table below was performed side by side and is based on following 45 control cells, 44 ALIX siRNA and 46 ALIX siRNA#2 transfected cells (from three separate experiments). Statistical significance is indicated as *p<0.05 and **p<0.01 (Student’s t-test).

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<th>prometaphase</th>
<th>metaphase</th>
<th>Anaphase</th>
<th>telophase</th>
<th>cytokinesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>con siRNA</td>
<td>16.8(0.9)</td>
<td>39.0(4.2)</td>
<td>8.4(0.5)</td>
<td>29.2(1.7)</td>
<td>41.0(2.4)</td>
</tr>
<tr>
<td>ALIX siRNA</td>
<td>21.6(1.4)**</td>
<td>73.6(14.8)*</td>
<td>7.6(0.7)</td>
<td>28.0(4.1)</td>
<td>60.4(10.7)*</td>
</tr>
<tr>
<td>ALIX siRNA #2</td>
<td>18.6(1.3)*</td>
<td>155.5(42.9)**</td>
<td>7.6(0.8)</td>
<td>33.6(8.4)</td>
<td>66.5(21)**</td>
</tr>
</tbody>
</table>

Functional interaction between ALIX and MAP1S as well as γ-TuRC was evaluated by microtubule sedimentation experiments in the presence or absence (kd) of ALIX in human cells. The corresponding full data set is now shown. The suppl. table lists many proteins based of the identification of one “significant” sequence without telling us about the sample origin (control or knockdown?). I still very much like the approach and I also understand that a microtubule sediment contains many more proteins than alpha tubulin and MAPs, but the way they data are presented (no legend that explains the categories, no clear assignment of ratios between control and knockdown, no information about replicates (just one experiment?); it would be good to label those proteins in the large table, which are shown in the small table in EV5A) does not fully support the conclusions drawn. Likewise, the new Western blot data (Fig. EV5B) neither show dramatic changes in the representation of MAP1S nor γ-TuRC on microtubules after ALIX knockdown. We have added a legend in Dataset EV1 explaining the underlying experiment and interpretation of the mass spectrometry analysis (including categories, how ratios are calculated and that the data are from one experiment) in accordance with the comment raised by the referee. We have also labeled the MAPs and γ-TuRC components presented in the small table in Figure EV5A in Dataset EV1 as explained in the dataset legend as well as in the manuscript (page 19) and Figure EV5A legend (page 47).

The dataset is representing one mass spectrometry analysis, which also might explain the putative discrepancy between the expected and observed results based on mass spectrometry and repeatedly Western blotting analysis, respectively. That being said, the trend indicated by the mass spectrometry data (reduced MT association of MAP1S and GCP3 and increased MT association of CLASP2) is validated in our Western blotting analysis. Importantly, in the quantification of the Western blots we calculated the amount of protein relative to tubulin intensity for each condition to adequately adjust for possible differences in MT-polymerization efficiency upon ALIX depletion. We do agree that our figure legend for could have been clearer in this respect and have updated it to clarify these issues (page 47).

Interaction of γ-TuRC with ALIX: the new pulldown experiment in Fig. 5F suggests interaction...
between the complex and ALIX: fine. A negative control, i.e. detection of any protein that does neither bind to GST nor to GST-ALIX, would strengthen this important result.

The pulldown experiment is performed in accordance to standard protocols (https://www.thermofisher.com/no/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/pull-down-assays.html). The negative control is agarose-beads coated with isolated GST, and as shown in Fig 5F, neither Dgrip91 nor γ-tubulin associate GST as efficiently as GST-ALIX, thus suggesting that ALIX interacts with Dgrip91 and γ-tubulin.

The authors now show a reduction of the centrosomal MAP1S signal after ALIX knockdown. This data would be stronger if a centrosomal marker was shown that was not altered and may have served as an internal control for centrosome position and quantification of the PCM and/or centrioles in general. The same applies to Fig. 5H showing a reduction in centrosomal γ-tub signals after ALIX knockdown.

We do agree with the referee that proper markers are essential when performing IF analysis. We now show new results in new Figure 5H where the centrosomes are marked with an antibody against the PCM-protein Pericentrin together with γ-tubulin. In contrast to γ-tubulin, ALIX-depleted cells show unchanged levels of centrosomal Pericentrin, thus implying that ALIX stabilizes specifically γ-tubulin at the centrosomes without affecting the general integrity of the PCM. The new results are described in the main text (page 16) and corresponding figure legend (page 39).

In Fig. 8A, MAP1S and γ-tubulin are shown. Ideally, we would have liked to co-stain the cells with anti-MAP1S and anti-Pericentrin in order to compare the reduced centrosomal localization of MAP1S to the unchanged Pericentrin. Unfortunately, both the MAP1S and Pericentrin antibodies are raised in rabbit and furthermore the ordered goat anti-Pericentrin antibody did not work satisfactorily. Therefore we have co-stained the cells with anti-MAP1S and anti-γ-tubulin to indicate the centrosome position, since the γ-tubulin levels are reduced and not completely abolished in ALIX-depleted cells.

The authors insist that GFP/YFP alone accumulate on spindles in an unspecified manner when overexpressed. How can we then interpret spindle localization of MAP1S or CLASP in then same panel? Just less protein expressed and then specificity regained? I find this hard to believe.

In the revised manuscript we present data of three new experiments in new Figure 8G-H in which the GFP fluorescence is very much retrieved/recovered by pH adjustment protocol since the very acidic formaldehyde solution used for fixation quenches the GFP fluorescence. Consequently, the YFP and GFP signals appear clearer, and even though some GFP and YFP apparently seem to stain the mid spindle in addition to the strong cytosol localization, YFP-MAP1S and GFP-CLASP2 are predominantly localizing to the spindle rather than the cytosol. Also with this updated protocol that improves the detection of YFP and GFP signals our new results confirm our previously presented results in old Figure 8G-H that ALIX-depleted cells exhibit significantly fewer and shorter astral MTs compared to control cells (p<0.05). Ectopic overexpression of MAP1S, but not CLASP2, significantly increases the length of the astral MTs in ALIX-depleted cells without affecting the number of MTs (p<0.05) (the fact that cells even with low YFP/GFP signals could be analyzed after pH adjustment might explain the partial rescue observed in these experiments compared to the experiments in old Figure 8G-H). The new data are presented in New Figure 8G-H and corresponding main text (pages 22-23) and figure legend (page 42). The experimental procedure is updated in the Supplementary Materials and Methods in the Appendix PDF (page 5).

Minor issues:

p. 16 top: Correspondingly ALIX depleted cells showed only 62...
We have in the updated manuscript inserted the missing word (cells) on (page 17 in revised text).

p. 45, fig. legend EV2: apoptosis as a consequence of metaphase arrest is not commonly refereed to as "mitotic exit".
We do agree that cells arrested in metaphase might have different fates. Therefore, we have rephrased the term in the table presented in Figure EV2J and replaced mitotic exit with metaphase death. The Figure legend is also corrected on page 45.
Thank you for submitting your final revised manuscript for our consideration. I have now had a chance to look through your files and to assess your responses to the remaining comments raised by original reviewer 3. I am happy to inform you that there are no further objections and that we have now accepted your study for publication in The EMBO Journal.
1. Data

The data shown in figures should satisfy the following conditions:
- The data were obtained and processed according to the field’s best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- Figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- Graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- If n, the individual data points from each experiment should be plotted and any statistical test employed should be justified.
- Source data should be included to report the data underlying graphs. Please follow the guidelines set out in the authorship guidelines on Data Presentations.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:
- A description of the experimental system investigated (e.g. cell line, species name).
- The assay(s) and method(s) used to carry out the reported observations and measurements.
- An explicit mention of the biological and chemical entity(ies) that are being measured.
- An explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- The exact sample size (n) for each experimental group/condition, given as a number, not a range.
- A description to understand whether the samples represent technical or biological replicates (including how many animals,itters, cultures, etc.).
- A statement of how many times the experiment was independently replicated in the laboratory.
- Definitions of statistical methods and measures:
  - Common tests, such as t-test (please specify whether paired or unpaired), simple q2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section.
  - Are tests one-sided or two-sided?
  - Are there adjustments for multiple comparisons?
  - Exact statistical test results, e.g., P values = x but not P values < x.
  - Definition of center values as median or average.
  - Definition of error bars as ± s.d. or ± s.e.m.

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

B. Statistics and general methods

1. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?

Experiments were performed on 4-day-old pupae to examine the spindle orientation and/or contractile intensity of a protein, and in general three independent experiments were performed, where at least 10 cells per experiment were analyzed. During determination of the spindle orientation in NBs and NPCs, as many metaphase cells as possible in 6-7 brains of each, respectively, dissected prior to experiment were analyzed, and at least three independent experiments were performed. During live cell imaging of dividing SCs used to determine spindle rotation speed in SOPs and percentage of mGSCs, SOPs were performed and as many dividing cells per pupal was possible were analyzed. During live cell imaging of migrating cells grown in 2D cultures, visualization of progress of label progression through the cell cycle was performed. For experiments presented in figures 9A and SEM, cells were performed by three persons. Independent observations of spindle orientation and position, from independent experiments performed and as many dividing cells per pupal was possible were analyzed. During determination of the Atad6 protein levels, per comparison, H2a-EGFP and control cells in metaphase were analyzed in 2-3 brains dissected per experiment (prepared at least twice). During determination of the Atad6 protein levels, per comparison, H2a-EGFP and control cells in metaphase were analyzed in 2-3 brains dissected per experiment (prepared at least twice). During determination of spindle orientation in NBs and NPCs, as many metaphase cells as possible in 6-7 brains of each, respectively, dissected prior to experiment were analyzed, and at least three independent experiments were performed. During live cell imaging of dividing SCs used to determine spindle rotation speed in SOPs and percentage of mGSCs, SOPs were performed and as many dividing cells per pupal was possible were analyzed. 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C. Reagents

4. To show that antibodies were profiled for use in the system under study (proxy and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile e.g., Antibodypedia (see link list at top right). 10homeo (see link list at top right).

5. Antibodies were profiled for use in the system under study (proxy and species), and the specific confirmation method was further validated in the current study (Fig. 1A and B). Rabbit anti-Acid was used originally in Cohnen A, 2005, C2C12 and verified here to recognize centroterminal MAb (Figure 1A, B). Rabbit anti-MAP4 (A2253, Dako), rabbit anti-Peripherin (p4406, Abcam), rabbit anti-

Variances were generally fairly similar. In a few cases with (moderately) skewed data, we also checked the results using log-transformation to obtain similar variances.

D. Animal Models

14. Report any restrictions on the availability (and/or on the use) of human data or samples.

15. For publication of patient photos, include a statement confirming that consent to publish was obtained.

16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under Reporting Guidelines. See also: NCI (see link list at top right) and UDI (see link list at top right) recommendations. Please confirm compliance.

E. Human Subjects

20. Identify the committee(s) approving the study protocol.

21. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

22. Could your study fall under dual use research of concern? Please check biosecurity documents and review manufacturer’s instructions. See author guidelines, under Reporting Guidelines. See also: NIH (see link list at top right) and UDI (see link list at top right) recommendations. Please confirm compliance.

F. Data Accessibility

28. Provide a “Data Availability” section at the end of the Materials and Methods. Listing the accession codes for data generated in this study and deposited in a public database (e.g., RNA-seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE P000082658). Please refer to our author guidelines for Data Deposition.

29. Data deposition in a public repository is mandatory for:

a. Protein, DNA and RNA sequences
b. Macromolecular structures
c. Crystallographic data for small molecules

30. Functional genomics data

31. Proteomics and molecular interaction

32. If deposition is strongly recommended, for any datasets that are central and integral to the study, please consider the journal’s data policy. If a structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under “Expanded View” or as an Addendum) or in a constructed repository such as Dryad (see link list at top right) or Figshare (see link list at top right).

33. Access to further animal and genetic datasets should be provided with a list of references as provided while requiring ethical obligations to the participants and relevant animal and ethical issues. If practical and possible and compatible with the individual consent agreement just in the study, each data should be deposited in one of the major public access controlled repositories such as DDBJ (see link list at top right) or EGA (see link list at top right).

34. De-identification of models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized formats (GML, CML) should be used instead of scripts (e.g., MATLAB). Authors are strongly encouraged to follow the MRWM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or SBML (see link list at top right). We also appreciated a computer-readable code is provided with the paper, it should be deposited in a public repository or included as supplementary information.

G. Dual use research of concern

36. Could your study fall under dual use research of concern? Please check biosecurity documents (see link list at top right) and list of dual use agents and toxins (DHHS/USA) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.