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An essential step of kinetochore formation controlled by the SNARE protein Snap29

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1st Editorial Decision 26 February 2016

Thank you for the submission of your manuscript entitled "An essential step of kinetochore formation controlled by the SNARE protein Snap29" and for your patience during the review process. We have now received and analyzed the reports from the two referees that accepted to evaluate your work, which I copy below.

As you can see from their comments, both referees are very supportive of your work, but point out to a number of significant concerns that will require your attention before your manuscript can be published in The EMBO Journal. I will not repeat here the referee concerns, which mostly refer to the conclusiveness of (particularly) your functional experiments and will defenitely require further experimental work, because I believe they are rather straightforward. In any case, please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems in addressing any of their concerns.

REFEREE REPORTS

Referee #1:

In this manuscript Morelli and colleagues study in Drosophila and human cells the potential role of the SNARE protein Snap29 at kinetochores in particular and in cell division in general. The authors show that Snap29 can be immunoprecipated with components of the KMN kinetochore complex, and that Snap29 itself localizes to kinetochores during mitosis in fly and human cells. The authors

further report that Snap29 depletion results in a mitotic delay and chromosome segregation errors both in Drosophila and in human. At the molecular levels this study furthers indicates that Snap29 depletion is associated with the loss of the KNL1 protein at kinetochores. Finally, the authors report that deregulation of Snap29 leads to neoplasia in fly tissues.

The strength of this study is that the main result of the study, the implication of the SNARE protein Snap29 in kinetochore function is novel, unexpected and intriguing, as it links for the first time the vesicle fusion machinery to cell division. The localization of Snap 29 at kinetochores is very well documented and based on solid data; however all the other functional data are based either on undocumented analysis, on results that were only reproduced once, or partially flawed conclusions. This very interesting study has the potential to have very strong impact in the field if all the data were solid, but at this stage it is significantly weakened by its technical quality. It would therefore greatly benefit from a substantial amount of work to provide the data that fully support the presented conclusions. Specifically:

While Figure 1 and 2 are of excellent quality, in Figure 3 the authors infer chromosome segregation defects and alignment defects after Snap29 from fixed cell imaging of Drosophila S2 cells. The problem with such an approach is that fixed cell imaging is not very informative to monitor a dynamic process such as cell division, as it is for example very difficult to differentiate between an early prometaphase cell from a metaphase cell that failed to align some of its chromosomes. If the authors want to describe the mitotic phenotype of Snap29 depletion in S2 cells, they will have to use live cell imaging of S2 cell expressing markers for chromosomes or kinetochores, which are available in the community. This would allow drawing much stronger conclusions.

In Figure 4, the authors do actually use live cell imaging of human U2OS cells, however their sample size is far too small. First, the authors report chromosome segregation errors in SNAP29-depleted cells, but they present no quantification of the error rate. Second, the authors analyze in their movies only 7 (wild-type) or 15 cells (Snap29 depletion). This is insufficient. Mitotic timing experiments often require 20 cells at minimum per experiment, and each experiment should be repeated at least 3 times, to make sure that the phenotype is reproducible. Such low numbers of cells cannot be representative for an entire cell population, and all experiments should be repeated multiple times independently. The authors should also be careful when interpreting the duration of the mitotic arrest in control and Snap29 depleted cells: I agree with the authors that Snap29-depleted cells have a functional SAC as nocodazole-treated cells arrest for more than 10 hours; however to conclude that they have a weaker SAC because the cells do not arrest for 18 hours unlike control depleted-cells is an over-interpretation, since this time also depend on factors that are independent of the SAC, such as degradation rate of the APC, Cyclin B1 levels etc. In the absence of more precise assays, such as Cyclin B1 degradation kinetics, the authors should stick to the more conservative conclusion, which is that the SAC is functional.

In Figure 5 the authors look at the stability of kinetochore-microtubule attachments and the status of the KMN network at kinetochores. First, the authors switch from U2OS cells to HeLa cells, without further explanation. Second, they present data from a cold-stable assay, but do not quantify their results. Moreover it is unclear how many times this experiment was repeated and how many cells were analyzed each time. Third, the presented Snap-29 depleted cells looks like an early prometaphase cell and the authors compare it to a control metaphase cell. At such a stage it is logical that many kinetochores will not have yet formed robust kinetochore-microtubule attachments in early prometaphase. How does the attachment look like in Snap29 depleted-cells that have a robust metaphase plate? In the subsequent experiments the authors measure inter-kinetochore distances (Fig. 5b and c). Their sample size is again far too small (e.g. only 1 prophase cell analyzed) and the experiments have not been repeated independently, therefore any statistics is not meaningful at this stage. Moreover, the Snap29-depleted cell shown in b) is by definition not in metaphase, since it has unaligned chromosomes. In fact the very low inter-kinetochore distances in these cells suggest that these kinetochores are simply unattached. Therefore, if the authors want to really test whether Snap29 depletion affects the ability of kinetochores to build tension, they should analyze only sister-kinetochore pairs that are aligned on the metaphase plate. In contrast, if the authors think that Snap29 depletion leads to many unattached kinetochores or that attachments per se are delayed, as implied by the images in Figure 5 a, b, and c, they should stain the cells for Mad2 or Mad1, which are very good markers for unattached kinetochores, or even better obtain from the J. Pines laboratory cell lines expressing endogenously tagged Venus-Mad2, which allows to look at

attachment in live. The presence of many unattached kinetochore would also fit with the spindle length measurements, which should also be repeated three times, since depletion of Nuf2R is known to lead to longer spindles (one should cite De Luca et al, 2002 in this context). Finally, the quantification in Figure 5l, should also be repeated independently, since the authors indicate that their measurements are built on only 10 cells, which I suppose represents one independent experiment.

The experiments in Figure 6 are nice, but the number of cells and the number of independent experiments are not indicated (the number of independent experiments is in fact not indicated in any of the figures), making it difficult to judge the significance of the results. Another potentially very interesting points is that the authors report that a mutant of Snap29 that lacks the first SNARE domain can complement the SNAP depletion, yet at the same time Figure 2 shows that the equivalent Drosophila mutant does not localize to kinetochores. This is a paradox, which is worth investigating in more detail: the authors should test whether the human Snap29 mutant lacking the first SNARE domain is present at kinetochores. If the mutant behaves like the Drosophila version, this would indicate that Snap29 is not required at kinetochores to ensure the loading of Knl1, which would lead to a re-interpretation of the model. This would not invalidate the presented data, but might suggest that the critical function of Snap29 in terms of Knl1 loading is not at kinetochores.

Finally the experiment presented in Figure 7 are nice and well executed, but at this stage the data is not strong enough to conclude that Snap29 mutations are sufficient to initiate tumorigenesis, since the authors did not perform an allograft culture, in which they have implanted their cells into the abdomen of an adult host. The authors should therefore either re-interpret their results in a more conservative manner, or perform such an allograft culture.

Referee #2:

The paper by Morelli et al describes a new role for the SNARE protein Snap29 in mitosis in Drosophila and human cells. This is a very interesting subject, as very little is known about the involvement of SNARE proteins in the cell cycle. Snap29 has been shown to be involved in trafficking pathways in interphase, but its role in cell division had not been characterized previously. More interestingly, the authors propose that these SNARE proteins (which have previously been associated with membrane trafficking) have a role in kinetochore formation.

In this manuscript the authors show that Snap29 interacts with Ndc80 and localizes to the outer kinetochore, using both confocal (showing colocalization with other centromere/kinetochore associated proteins) and electron microscopy in S2 cells. Analysis of dividing ephithelial cells (imaginal discs) allowed them to show that the localization depends on Mis12/Kln/Ndc80 but not RZZ; they also could investigate which regions of the protein are important for recruitment to the kinetochore: using forms of Snap29 without NPF motif or either of the SNARE domains they show that the first SNARE domain mediates recruitment.

Next the authors show that Snap29 promotes accurate chromosome segregation by a) mediating recruitment of Knl1 and b) ensuring stable MT attachments. The localization of Knl1 requires Snap 29 (human or Drosophila) and it is prevented by a point mutation that blocks Snap29 release from SNARE fusion complexes. This mutant causes ectopic Knl1 recruitment to trafficking compartments.

This is a very nice study that elegantly takes advantage of two different model systems to investigate a relatively new aspect of chromosome biology. My main concerns are about some aspects of the quality of the microscopy results shown-which are crucial to support the authors' claims- and the statistical analysis of some of the results. As a general comment, in all the experiments involving immunofluorescence microscopy analysis the authors should show the different channels in black and white to make visualization easier (in particular it is very difficult to distinguish clearly any figure shown in blue over black background!). Whenever possible the authors should also include high magnification insets of the structures studied (in most cases kinetochores) and use arrows to clearly indicate subcellular positions. Single Z-sections can be useful to show specific details but in general projections of Z-sections should be used for quantification purposes.

Specific points:

- In figure 1C-F the authors need to show clearly in all panels examples of isolated chromosomes/kinetochores similarly to the one shown in 1e. This is important to support the specific localization of Snap29 with respect to the inner/outer kinetochore proteins

- Unspecific centrosomal staining is not uncommon when using polyclonal sera, additional evidence should be shown to support the centrosomal localization is real.
(figure 1f).

- The localization of Snap29 in anaphase/telophase is not clear from the figures (Suppl 1a) The centrosomal staining is not obvious either.

- Figure 2, I have similar suggestions as above; in the changes suggested are even more necessary for non-specialist readers. The scale bars are missing. Use higher magnification insets and arrows pointing to the relevant structures. Some of the panels are missing the QPCR quantification data (% of expression). These experiments also require quantification of the Snap29 protein signal (normalized using an appropriate marker).

- The experiments described in Suppl fig S3 need to show levels of depletion of the protein/mRNA, separate b/w panels -at least for Snap29-, and quantification of the levels of Snap29 showing appropriate statistical analysis.

- Snap29 depleted S2 cells show abnormalities in chromosome congression and segregation. In order to demonstrate that Snap29 promotes accurate chromosome segregation these defects need to be categorized and properly quantified. In figure 3, similar suggestions apply as for previous figures. The level of depletion of Snap29 needs to be quantified. To analyze the mitotic index/distribution of phases of mitosis these experiments need to be replicated and subject to adequate statistical analysis (significance, p-values, etc when necessary). The authors should quantify cells in prometaphase as a separate category, and all the different defects described need to be categorized and quantified.

- In Figure 5, the prometaphase phenotype in panel A needs to be properly quantified. The experiment in panel B requires clarification: were the inter-kinetochore distances measured in unaligned/aligned chromosomes? In which phase of mitosis exactly? Once this is clearly categorized, Panel C should include statistical analysis to show if the differences (between similar phases/aligned or unaligned chromosomes) are significant.

- Similarly, in Figure 5d, although some attempt has been made to quantify the different phenotypical categories an adequate analysis is missing. HeLa cells in my experience never show 100% of mitotic cells with normal spindles, maybe the sample size is not big enough.

- In Figure 5F-k, please show high magnification insets of kinetochores. In panel 5I - does partial mean reduced levels or only present in some chromosomes or both? Show error bars/statistical analysis.

- The experiments described in figure 7 show evidence of a role for Snap29 as a tumor suppressor. Although preliminary, these are quite exciting findings that will surely be the subject of a follow-up study.

1st Revision - authors' response 02 June 2016

Response to reviewers

We thank the reviewers for providing very constructive criticism of our work. We are delighted that they define it a "very interesting study [that] has the potential to have very strong impact in the field" and a "very nice study that elegantly takes advantage of two different model systems to investigate a relatively new aspect of chromosome biology". As you will see in the point-by-point response below (**bold**), we have addressed the criticism in full and we are pleased to present an improved version of the manuscript. Briefly, we have repeated and quantified most of the experiments, increasing dramatically sampling numbers, as requested by the reviewers. We present a detailed account of the quantification methods and statistics in the Material and Methods and Figure Legends sections. Also, we now present a time-lapse analysis of S2 cells and allograft experiments, as suggested by the reviewer 1. Moreover, we now analyze Snap29 localization in *Drosophila* cells by super-resolution, and we have separated channels and included high magnification insets for most of the experiments, as suggested by the reviewer 2. We hope that the reviewers will find the manuscript in its present form suitable for publication in *EMBO Journal*.

Thomas Vaccari

Response to reviewer 1:

1. While Figure 1 and 2 are of excellent quality, in Figure 3 the authors infer chromosome segregation defects and alignment defects after Snap29 from fixed cell imaging of Drosophila S2 cells. The problem with such an approach is that fixed cell imaging is not very informative to monitor a dynamic process such as cell division, as it is for example very difficult to differentiate between an early prometaphase cell from a metaphase cell that failed to align some of its chromosomes. If the authors want to describe the mitotic phenotype of Snap29 depletion in S2 cells, they will have to use live cell imaging of S2 cell expressing markers for chromosomes or kinetochores, which are available in the community. This would allow drawing much stronger conclusions.

We thank the reviewer for suggesting such an experiment, that we have now performed 3 times. We have included 1 of the experiments as a representative example in revised Figure 3a-c. We are happy to supply data of the other two experiments, for this and all the other repeated experiments (see below), upon request. As for the other new or repeated experiments, we have revised the material and method section and added a new paragraph "Measurements" with detailed explanation of the quantifications and statistical treatments. By time lapse microscopy, we observe that S2 cells stably expressing spindle and mitotic DNA markers (from Sylvia Erhart lab), when depleted of Snap29 for 96 hrs fail to form a normal metaphase plate and, more rarely, display other mitotic defects. We have quantified these by analyzing the behavior of >40 cells/sample and present representative movies as supplementary data (movie 1-2). We note that defects are very reminiscent of those observed in human cells. The relevant part of the text has been amended to include the new experiment. Considering the reviewer criticism, we have decided to remove the original experiment in fixed cells.

2. In Figure 4, the authors do actually use live cell imaging of human U2OS cells, however their sample size is far too small. First, the authors report chromosome segregation errors in SNAP29-depleted cells, but they present no quantification of the error rate.

In consideration of the fact, correctly pointed out by the reviewer, that we present no quantification of the error rate, we have toned down the description of the time-lapse experiment in U2OS cells. On page 9, we now say: "compared to control,

SNAP29 knocked-down (SNAP29 KD) cells occasionally divide with chromosomes incorrectly aligned to the metaphase plate, generating daughter cells with more than one nucleus (Fig. 3d-e; movie 3-5).”

Second, the authors analyze in their movies only 7 (wild-type) or 15 cells (Snap29 depletion). This is insufficient. Mitotic timing experiments often require 20 cells at minimum per experiment, and each experiment should be repeated at least 3 times, to make sure that the phenotype is reproducible. Such low numbers of cells cannot be representative for an entire cell population, and all experiments should be repeated multiple times independently.

As requested, we have now repeated 3 more times independently and quantified the mitotic delay using >20 cells/sample. In revised Fig. 3, we present the quantification of the mitotic delay of a representative new experiment. We have amended the relevant part of the text and provide numbers and quantification methods in the revised of Fig. 3.

3. The authors should also be careful when interpreting the duration of the mitotic arrest in control and Snap29 depleted cells: I agree with the authors that Snap29-depleted cells have a functional SAC as nocodazole-treated cells arrest for more than 10 hours; however to conclude that they have a weaker SAC because the cells do not arrest for 18 hours unlike control depleted-cells is an over-interpretation, since this time also depend on factors that are independent of the SAC, such as degradation rate of the APC, Cyclin B1 levels etc. In the absence of more precise assays, such as Cyclin B1 degradation kinetics, the authors should stick to the more conservative conclusion, which is that the SAC is functional.

We have repeated the experiment in HeLa cells (now in Fig. 4d), confirming the original data. However, we agree with the reviewer that the time of arrest might depend on other factors. Thus, as suggested, we have rephrased and now state on page 10: “In continuous presence of Nocodazole, SNAP29 KD cells exit from mitosis earlier than control cells (Fig. S4d), suggesting either that the SAC is inefficient, or that other factors that control mitotic timing are affected by SNAP29 depletion”.

4. In Figure 5 the authors look at the stability of kinetochore-microtubule attachments and the status of the KMN network at kinetochores. First, the authors switch from U2OS cells to HeLa cells, without further explanation.

We switched to HeLa cells for practical reasons, based on the fact that all the mitotic phenotypes of SNAP29 depletion in U2OS cells are observed in HeLa. This is shown not only for formation of cells with micronuclei and delay (shown in revised Fig. 3d-e for U2OS cells and in revised Fig. S4a, c for HeLa in fixed cells), but also for adaptation time (now shown in HeLa cells in revised Fig. S4d; originally in U2OS in Fig. 4E) and for chromosome alignment at metaphase (now shown in revised Fig. 3d for U2OS cells and in revised Fig. 4c for HeLa; please see point 6 for explanation of the new experiment in Fig. 4c).

5. Second, they present data from a cold-stable assay, but do not quantify their results. Moreover it is unclear how many times this experiment was repeated and how many cells were analyzed each time.

As requested, we have now repeated the experiment 3 more times and quantified the results (please see also point 6). In revised Figure 4a-b, we now present representative examples and quantification of 3 independent experiments based on 20 cell/sample, in which we have analyzed 10 KTs/cell. We have performed this experiment and the other of revised Fig. 4 under milder KD conditions compared to the original experiments to be able to identify metaphases in depleted cells (see point 6 below for details).

6. Third, the presented Snap-29 depleted cells looks like an early prometaphase cell and the authors compare it to a control metaphase cell. At such a stage it is logical that many kinetochores will not have yet formed robust kinetochore-microtubule attachments in early prometaphase. How does the attachment look like in Snap29 depleted-cells that have a robust metaphase plate?

We agree with the reviewer that our original data did not resolve whether the depleted cells are in fact in prophase or in metaphase, as for the most part they are unable to form a recognizable metaphase plate. To overcome such ambiguity, we have decided to use less strong knock down condition. (we have now analyzed cells

treated with siRNA for 48 hrs rather than 72 hrs). In this condition, depleted cells still form a recognizable metaphase plate with many chromosomes scattered around the pole that are positive for MAD1 (please see revised Fig 4a-c). We counted attachment in control and KD cells and found that in KD cells in metaphase roughly half of the KTs counted are unattached and MAD1-positive, while in control metaphase cell the vast majority are attached and MAD1-negative, indicating the KT - MT binding is not properly formed or stabilized.

7. In the subsequent experiments the authors measure inter-kinetochore distances (Fig. 5b and c). Their sample size is again far too small (e.g. only 1 prophase cell analyzed) and the experiments have not been repeated independently, therefore any statistics is not meaningful at this stage. Moreover, the Snap29-depleted cell shown in b) is by definition not in metaphase, since it has unaligned chromosomes. In fact the very low inter-kinetochore distances in these cells suggest that these kinetochores are simply unattached. Therefore, if the authors want to really test whether Snap29 depletion affects the ability of kinetochores to build tension, they should analyze only sister-kinetochore pairs that are aligned on the metaphase plate.

We have repeated the experiment 3 times independently using milder KD conditions (see above) and measured 10 chromosomes/cell in 10 cells/sample (revised Fig. 4c-d). Compared to the original observation, we find that not only unaligned metaphase KT display significantly short inter-KT distances but also aligned ones (albeit to a lesser extent). These new data suggest that upon depletion of SNAP29 some chromosome are able to bind MT stably enough to reach the metaphase plate and develop some tension, while others don't. In the process of repeating the experiment, we realized that the imageJ plugin for measurement of inter-KT distance contained a conversion error that lead to gross over-representation of the distance (3 microns instead of 1.2 in control cells!). We have corrected the mistake and now show accurate measurements. We are very grateful to the reviewer for suggesting to repeat this experiment that allowed us to spot the glitch.

8. In contrast, if the authors think that Snap29 depletion leads to many unattached kinetochores or that attachments per se are delayed, as implied by the images in Figure 5 a, b, and c, they should stain the cells for Mad2 or Mad1, which are very good markers for

unattached kinetochores, or even better obtain from the J. Pines laboratory cell lines expressing endogenously tagged Venus-Mad2, which allows to look at attachment in live.

We thank the reviewer for the insightful suggestion. We have now have added MAD1 stains as requested (now in revised Fig. 4a-b). We observe that MAD1 positive KT tend to be those that of chromosome that are not aligned to the metaphase plate in KD cells, in agreement with the evidence shown in revised Fig. 4.

9. The presence of many unattached kinetochore would also fit with the spindle length measurements, which should also be repeated three times, since depletion of Nuf2R is known to lead to longer spindles (one should cite De Luca et al, 2002 in this context).

We have repeated the experiment 3 times independently and now show one representative example of the new experiment in revised Fig. 4e-f. We have moved the categorization of spindle phenotypes, which we have also amended (please see response to reviewer 2, point 13), to the text (Page 10-11) and we now cite De Luca et al as requested. We apologize for the missing reference.

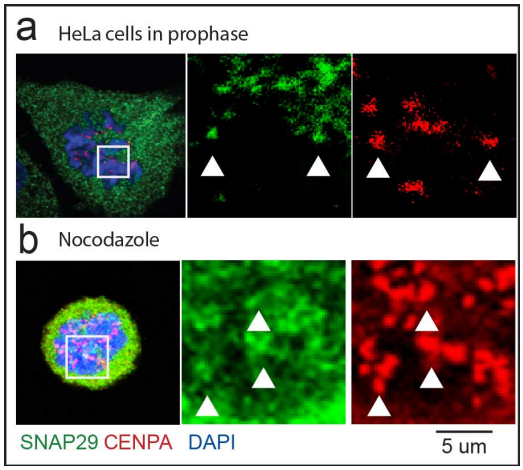
10. Finally, the quantification in Figure 5l, should also be repeated independently, since the authors indicate that their measurements are built on only 10 cells, which I suppose represents one independent experiment. The experiments in Figure 6 are nice, but the number of cells and the number of independent experiments are not indicated (the number of independent experiments is in fact not indicated in any of the figures), making it difficult to judge the significance of the results.

We have repeated the experiments 3 times independently in each counting at least 100KTs/cell out of 10 cells/sample and now show quantification in revised Fig. 5f and 6d (please see also figure legends and material and methods for details). To simplify the representation of the quantified data we consider only two phenotypic categories, 'signal' (corresponding to the original 'full' and 'partial') and 'no signal' (corresponding to the original 'absent'). The quantifications and statistical analyses support nicely our original conclusions.

11. Another potentially very interesting points is that the authors report that a mutant of Snap29 that lacks the first SNARE domain can complement the SNAP depletion, yet at the same time Figure 2 shows that the equivalent Drosophila mutant does not localize to

kinetochores. This is a paradox, which is worth investigating in more detail: the authors should test whether the human Snap29 mutant lacking the first SNARE domain is present at kinetochores.

Considering that we express constructs in HeLa cells transiently, and thus at variably high levels, it is possible that the rescue of KT formation in depleted human cells expressing a SNAP29 lacking the first SNARE domain might be aided by the excess of mutant protein. Despite this, we agree with the reviewer that it would be nice to show also the localization of human SNAP29. However, the antibody against human SNAP29 that we have generated works nicely for Western blot and in cells overexpressing SNAP29 at high levels , especially for mutant forms like the Q12 that are trapped at membranes. (see for example revised Fig. S4b and 6e). However, it doesn't work well to visualize endogenous protein levels by immunofluorescence. In these condition, we observe a minor proportion of signal at the KT, that is a bit enhanced by nocodazole treatment (please see reviewer figure below). We are now generating stable HeLa cell lines expressing low levels of tagged human SNAP29 and CRISPR HeLa cell that do not express SNAP29 for such analyses and to control for antibody specificity. We will present these data in a follow up study at a later time.



12. If the mutant behaves like the Drosophila version, this would indicate that Snap29 is not required at kinetochores to ensure the loading of Knl1, which would lead to a re-interpretation of the model. This would not invalidate the presented data, but might suggest that the critical function of Snap29 in terms of Knl1 loading is not at kinetochores.

We agree with the reviewer that in absence localization data in human cells of this possibility exist. We have modified the discussion to reflect this and now say: "The ability of KNL1 to interact with a SNAP29 that cannot be released from SNARE complex, both suggest that the interaction of KNL1 with SNAP29 might occur on the side of the SNARE domain that is not occupied by the Vamp. These data also suggest that SNAP29 could act on KNL1 also prior to nuclear entry and KT localization." We thank the reviewer for pointing out this possibility that helped avoiding over interpretation of our data.

13. Finally the experiment presented in Figure 7 are nice and well executed, but at this stage the data is not strong enough to conclude that Snap29 mutations are sufficient to initiate tumorigenesis, since the authors did not perform an allograft culture, in which they have implanted their cells into the abdomen of an adult host. The authors should therefore either re-interpret their results in a more conservative manner, or perform such an allograft culture.

As suggested by the reviewer, we have performed the allograph experiment following the published reference procedure (Rossi F et al Nature protocols 2015). We could not label genetically the tumors generated in the eye disc by recombination over a cell lethal chromosome, or analyze multiple allograft passaging in the time-frame of the revision. However, using a host expressing nuclear His2Av-mRFP, we were able to recover unlabeled tumor tissue from 12 to 28 days after implant from 9 animals, starting from both Snap29-B6-21/cell lethal tissue (n=4) or Snap29-B6-21/cell lethal p35 (n=5). We measured the average area of the explanted tissue and compared it with the average area of the tissue before allografting and found differences that suggest that the allografted tissue is able to grow into the hosts, irrespective of the tumor genotypes. Also, we find the the explanted allografted tissue is positive for pH3, suggesting cells can divide in the host a long time after the explant (revised Fig. S7f-h; please see exact genotypes in revised Table S1). Because the allografted tissue survives and grow but does not behave as aggressively as other allografts in the literature (see for example *numb* in Caussinus et al 2005), we have removed mention of tumor suppression in the abstract and result section. We refer to Snap29 as supporting tissue formation (see for example page 13-14) and only refer to tumor suppression as a possibility once in the discussion.

Response to reviewer 2:

1. My main concerns are about some aspects of the quality of the microscopy results shown-which are crucial to support the authors' claims- and the statistical analysis of some of the results. As a general comment, in all the experiments involving immunofluorescence microscopy analysis the authors should show the different channels in black and white to make visualization easier (in particular it is very difficult to distinguish clearly any figure shown in blue over black background!).

We apologize for the presenting less than completely clear images. As requested we now present all single channels in black and white in most figures.

2. Whenever possible the authors should also include high magnification insets of the structures studied (in most cases kinetochores) and use arrows to clearly indicate subcellular positions.

We now present insets in most figures.

3. Single Z-sections can be useful to show specific details but in general projections of Z-sections should be used for quantification purposes.

As suggested, we have used Z-stacks for quantification purposes in all the repetition experiments for quantification (see for example revised Fig 2 and revised Material and methods).

Specific points:

4. In figure 1C-F the authors need to show clearly in all panels examples of isolated chromosomes/kinetochores similarly to the one shown in 1e. This is important to support the specific localization of Snap29 with respect to the inner/outer kinetochore proteins

To be more precise about describing Snap29 localization at the kinetochore relative to other components, in revised Fig. 1 we now show super-resolution imaging obtained by STED microscopy. The new data show clearly that Snap29 is external to

CenpC and slightly internal/partially overlapping with Spc105R. Please see also revised text on page 6.

5. Unspecific centrosomal staining is not uncommon when using polyclonal sera, additional evidence should be shown to support the centrosomal localization is real.
(figure 1f).

Because we are not showing any follow up data on Snap29 localization at the centrosome, we have decided to not present the localization in question. We will consider whether to present it together with other data in a follow up study focusing on centrosomal function at a later time.

6. The localization of Snap29 in anaphase/telophase is not clear from the figures (Suppl 1a)

We apologize for presenting less than optimal images. We have replaced them with a better example in revised Fig S1a.

7. Use higher magnification insets and arrows pointing to the relevant structures.

We now show higher magnification insets in most figures and added more arrows, when needed. We hope that with the single B/W channels and the higher mag images structures of interest are now more visible.

8. The centrosomal staining is not obvious either.

Please see response to point 5.

9. Figure 2, I have similar suggestions as above; in the changes suggested are even more necessary for non-specialist readers. The scale bars are missing.

- Some of the panels are missing the QPCR quantification data (% of expression).
- These experiments also require quantification of the Snap29 protein signal (normalized using an appropriate marker).

We regret the oversights. We have now repeated the experiment 3 times independently, and present a representative example of the new experiment in revised Fig. 2. This now includes full quantification of Snap29 levels based on z-stacks in all samples, supporting the original conclusions. To unambiguously identify kinetochores in dividing cells, we costained to detect the invariant kinetochore component CenpC and the mitotic marker pH3. The quantification graph includes knock-down levels by QPCR (please see also revised material and methods for details on the method of quantification). As requested, the presentation now includes higher magnifications, single channels in black and white and scale bars. We have excluded the Spindly knock-down because, upon repeating the experiment, we did not obtain consistency across the 3 repetitions. Because Spindly is not central to the story, we have removed mention of it.

10. The experiments described in Suppl fig S3 need to show levels of depletion of the protein/mRNA, separate b/w panels -at least for Snap29-, and quantification of the levels of Snap29 showing appropriate statistical analysis.

We have revised extensively Fig. S3 by adding quantification of the Snap29 signal relative to CenpC, obtained similarly to revised Fig. 2 (see above). Also, we have split channel as requested. Finally, we have removed the Ndc80 knock down data that recapitulate what we already show *in vivo* in revised Fig. 2.

11. Snap29 depleted S2 cells show abnormalities in chromosome congression and segregation. In order to demonstrate that Snap29 promotes accurate chromosome segregation these defects need to be categorized and properly quantified. In figure 3, similar suggestions apply as for previous figures. The level of depletion of Snap29 needs to be quantified. To analyze the mitotic index/distribution of phases of mitosis these experiments need to be replicated and subject to adequate statistical analysis (significance, p-values, etc when necessary). The authors should quantify cells in prometaphase as a separate category, and all the different defects described need to be categorized and quantified.

We have repeated the experiment as requested by using time lapse analysis using S2 expressing mitotic and spindle markers (now in revised Fig. 3). Please see response to Reviewer #1 point 1 for details.

12. In Figure 5, the prometaphase phenotype in panel A needs to be properly quantified. The experiment in panel B requires clarification: were the inter-kinetochore distances measured in unaligned/aligned chromosomes? In which phase of mitosis exactly? Once this is clearly categorized, Panel C should include statistical analysis to show if the differences (between similar phases/aligned or unaligned chromosomes) are significant.

We have repeated all the experiments in former Fig. 5a-e (now in revised Fig. 4) in a different way to address these questions. Please see reviewer #1 point 4-6 for details.

13. Similarly, in Figure 5d, although some attempt has been made to quantify the different phenotypical categories an adequate analysis is missing. HeLa cells in my experience never show 100% of mitotic cells with normal spindles, maybe the sample size is not big enough.

We went back to images of the original experiment and categorized more cells from the control sample. Counting 102 control cells, we realized that indeed 9.8% (10 cells) show a tripolar spindle, while 90.2% (92 cells) show a normal bipolar spindle. We have now updated the number of control cells and moved the description of the experiment from the figure to the text (page 10-11) to make space for the repetition of the quantification of the spindle length (now in revised Fig. 4e-f). We thank the reviewer for this comment that prevented us from under-representing the defects of control HeLa cells.

14. In Figure 5F-k, please show high magnification insets of kinetochores.

As requested, we now present insets for each panel of revised Fig. 5 and 6.

15. In panel 5l - does partial mean reduced levels or only present in some chromosomes or both? Show error bars/statistical analysis.

We apologize for the lack of clarity. In the original experiment partial meant reduced levels at single kinetochores. We had used an arbitrary threshold of pixel intensity (150) above which a single KT was considered to have "full" signal. If the signal was visible but below 150, we considered it "partial". KT with no signal visible were counted in the category "absent". We have now repeated the experiments 3 other

times, in each experiment counting 100-200 KT/sample of 10 cells, as requested by reviewer 1 (see also response to reviewer 1, point 10). For clarity in presenting the new quantified experiment (revised Fig. 5-6), we considered only "signal" (former full and partial categories) and "no signal" (formerly "absent").

16. The experiments described in figure 7 show evidence of a role for Snap29 as a tumor suppressor. Although preliminary, these are quite exciting findings that will surely be the subject of a follow-up study.

We are delighted that the reviewer defines our data in figure 7 "exciting findings". To strengthen the data, upon suggestion of reviewer #1, we now present also allograft experiments (revised Figure S7; please see reviewer #1 point 13 for details).

2nd Editorial Decision 23 June 2016

Thank you for the submission of your revised manuscript to The EMBO Journal. As you will see below, your article was sent back to the original referees, who now consider that you have properly dealt with most of their main concerns originally raised in the review process, although two points raised by referee #1 will still need your attention. These concerns mainly refer to the presentation of data in the paper, but need to be addressed, experimentally if necessary.

REFEREE REPORTS

Referee #1:

The authors have improved the manuscript and present a more thorough study based on better assays, which is positive. Nevertheless, there are still 2 remaining major points that preclude publication at this point.

1. One of the original criticisms, was that some results were supported by only 1 independent experiment, which does not allow to test whether Snap29 depletion leads to a reproducible phenotype. The authors have now performed 3 independent experiments for all experiments, yet in many figures they still only show the representative quantification of 1 experiment along with the statistical analysis for that 1 experiment. This is not the proper standard. Such a representation only shows that Snap29 depletion gave a phenotype on that particular day. The correct standard is to show the average for a minimum of 3 independent repetitions including the standard error of the mean, as this will show whether a particular depletion gives a reproducible phenotype (3 being a minimum). Only then will the reader be able to estimate the strength and reproducibility of Snap 29 depletion. Moreover, the statistical analysis has to performed on the means or medians of the 3 independent experiments. In this context I recommend to read Cumming et al, JCB, 2007.

2. the second concern is that instead of quantifying the chromosome segregation error rate in U2OS cells depleted of Snap29, the authors have now toned down their conclusion, and they report that they observe occasional errors without quantification. However, the presence of chromosome segregation errors is the justification for the whole second part of the paper, and is the key element to show the physiological relevance of Snap29. Therefore this information is crucial for the reader and it cannot be omitted.

Referee #2:

This is a considerably improved version of the manuscript. The authors have addressed all my original criticisms successfully. They include new data that contributes to support the main conclusions (in particular the super-resolution analysis of Snap29 localization and time lapse experiments). The quantifications and statistical analysis included in the new version are appropriate. I have no further queries for the authors, I think the manuscript is now suitable for publication.

2nd Revision - authors' response 12 August 2016

Response to reviewer 1:

1. One of the original criticisms, was that some results were supported by only 1 independent experiment, which does not allow to test whether Snap29 depletion leads to a reproducible phenotype. The authors have now performed 3 independent experiments for all experiments, yet in many figures they still only show the representative quantification of 1 experiment along with the statistical analysis for that 1 experiment. This is not the proper standard. Such a representation only shows that Snap29 depletion gave a phenotype on that particular day. The correct standard is to show the average for a minimum of 3 independent repetitions including the standard error of the mean, as this will show whether a particular depletion gives a reproducible phenotype (3 being a minimum). Only then will

the reader be able to estimate the strength and reproducibility of Snap 29 depletion. Moreover, the statistical analysis has to performed on the means or medians of the 3 independent experiments. In this context I recommend to read Cumming et al, JCB, 2007.

We now present quantifications based on at least 3 experiments and graphed as means and standard error of the mean, as requested (See revised figure 2, 3, 4, S3, S4). Also, we have added in the figure legends the average number of objects quantified per experiment. Finally, we have substituted a couple of panels in Fig. 2 and Fig. 3 to reflect the updated quantification or improve clarity. In Fig. 2, we have substituted Rod KDs with Zw10 KD. Both are components of the RZZ complex and show the same phenotype. We had run the samples in parallel in all the experiments but we have realized to have for Rod only an n of 2 experiments for technical reasons, so we included Zw10 for which we have an n>3. In Fig. 2, we have decided to show a larger field of dividing S2 cells to illustrate all defects observed (corresponding movies are now presented as Movie1 and 2).

2. the second concern is that instead of quantifying the chromosome segregation error rate in U2OS cells depleted of Snap29, the authors have now toned down their conclusion, and they report that they observe occasional errors without quantification. However, the presence of chromosome segregation errors is the justification for the whole second part of the paper, and is the key element to show the physiological relevance of Snap29. Therefore this information is crucial for the reader and it cannot be omitted.

We now present quantification of the mitotic defect in U20S cells based on 3 experiments, in which we analyzed movies for an average of 28 cells/experiment for control cells and of an average of 32 cells/experiment for SNAP29 KD cells. We found that 59±20% of the SNAP29 KD cells divided aberrantly to form 1 or more micronuclei, while of only 6±3% of the control cells did so. The P value is 0.037, indicating the that the presence of errors is significant. These data are now presented in the text at page 9.

3rd Editorial Decision 16 August 2016

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

EMBO PRESS

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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Materials and methods page 27 and legends of figure 2-6 and appendix supp fig. 4
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	the data were acquired and analyzed by the multiple investigators including the PI and some of the experiments were performed blind.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	yes, see legends of figure 2-6 and appendix supp fig. 4 for details
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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Material and methods page 18

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8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Genotypes of invertebrate animals are provided in Table 1 of the appendix. Rearing conditions are described in material and methods page 18
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