EMBO reports

RAB11A and RAB11B control mitotic spindle function in intestinal epithelial progenitor cells of mice

Ivor Joseph, Juan Flores, Victoria Farrell, Justin Davis, Jared Bianchi-Smak, Qiang Feng, Sayantani Goswami, Xiang Lin, Zhi Wei, Kevin Tong, Zhaohui Feng, Michael Verzi, Edward Bonder, James Goldenring, and Nan Gao
DOI: 10.15252/embr.202256240

Corresponding author(s): Nan Gao (ngao@andromeda.rutgers.edu)

Review Timeline:

Submission Date:	5th Oct 22
Editorial Decision:	9th Nov 22
Revision Received:	8th May 23
Editorial Decision:	2nd Jun 23
Revision Received:	9th Jun 23
Editorial Decision:	19th Jun 23
Revision Received:	20th Jun 23
Accepted:	27th Jun 23

Editor: Ioannis Papaioannou

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **9th Nov 2022**

Dear Nan,

Thank you for the submission of your research manuscript to EMBO reports. It has now been seen by three experts in the field, and I have already sent you their detailed reports (they are appended again below). I would also like to thank you for our discussion on their comments yesterday.

Referee #1 acknowledges that the work is novel, comprehensive and significant, and only has a few suggestions for minor improvements. Referee #3 agrees that the findings are potentially interesting, but also raises several technical concerns that must be addressed. Referee #2 points out that the mechanistic investigation linking Rab11 proteins to Kif11 is not conclusive, and that it remains unclear whether the observed effects are direct and the interactions specific. We agree that further experimental investigation will be necessary for stronger support of these conclusions. Furthermore, the referees provide a number of additional suggestions for the improvement of the study and the manuscript, which should be addressed.

Given these constructive comments and your willingness to address the criticisms of the referees, we would like to invite you to revise your manuscript along the lines you suggested with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript. If you have any questions or comments, we can also discuss the revisions in a video chat, if you like.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we usually recommend a revision within 3 months (February 8th). Please discuss with me the revision progress ahead of this time if you require more time to complete the revisions.

*****
IMPORTANT NOTE:
We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section providing access to data deposited in public databases is missing.

2) Your manuscript contains statistics and error bars based on n=2. Please use scatter plots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.
*****

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper unless you opt out of this (please see below for further information).

4) a complete author checklist, which you can download from our author guidelines (<https://www.embopress.org/page/journal/14693178/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>)

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).
Specifically, we would kindly ask you to provide public access to the following datasets/data:
- RNA sequencing data
- Proteomic (mass spectrometry) data

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below (see also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

8) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the new policy (<https://www.embopress.org/competing-interests>) and update your competing interests statement if necessary. Please name this section 'Disclosure and competing interests statement' and place it after the Acknowledgements section.

9) Figure legends and data quantification:
The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter plots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please also include scale bars in all microscopy images.

10) At EMBO Press we ask authors to provide source data for the main and EV figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as

follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>.

12) Please also note our reference format:
<http://www.embopress.org/page/journal/14693178/authorguide#referencesformat>.

13) We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section, which should be removed from the manuscript. Please use the free text box to provide more detailed descriptions. See also guide to authors:
<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>.

14) As part of the EMBO publications' Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You can opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

You can use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

This manuscript describes the effects of knockout of both Rab11a and Rab11b in the mouse intestine. This work is novel because the ability to knock out both forms of Rab11 in vivo has been impossible due to embryonic lethality. Even so, double knockout of these proteins is rapidly lethal, indicating both their redundancy and importance. This is a comprehensive study that analyzes the effects on intestinal architecture, as well as effects on the intestinal epithelium itself. The impacts of Rab11 knockout are robust, with villus blunting, changes in enterocyte gene expression, changes in secretory lineages, and effect on stem cells. Strikingly, there is a substantial defect in the cell cycle, which is reflected in aberrant bipolar spindle assembly and arrested cell cycle. Co-immunoprecipitation finds interaction of Rab11 with multiple proteins known to be involved in cell division, common to both Rab11a and Rab11b, with Kif11 being ranked highest. Further experiments defined the importance of this interaction for normal spindle pole assembly and subsequent successful mitosis. Overall, this study that strongly links the cell autonomous effects of Rab11 knockout on intestinal epithelial proliferation through interaction with Kif11.
Minor concerns:
Figure 1A. All of the colors in the legend are not discernible on the figure. While one can infer that the blue and orange are under the black line, it would be preferable to represent them all.
Figure 1P. The image quality in this panel is very low.
Figure 2C. While the text states that at 2d in the DKO there is a difference in Ki67 labeling, there is no statistical analysis of this time point.
Figure 2F is redundant with 2G, H.
In general, the fluorescent images are presented as a single merged panel. It would be useful to have the split channels together with the merged images to allow the reader to assess colocalization.

Figure 6O. The propridium iodide staining is relatively uninterpretable. It is clear that there is more, but assigning the staining to any population is difficult. 2D enteroids would be better suited to this experiment.

Referee #2:

In the present manuscript the authors report a new role for Rab11 in bipolar spindle formation in mouse epithelia cell division, which they propose is mediated by the Kinesin-5 family member, Kif11. Loss of both Rab11 isoforms (a and b) causes cell cycle defects leading to apoptosis, which ultimately causes a loss of differentiated epithelial cells. They postulate that this is a novel mechanism for cell division and tissue homeostasis.

The manuscript contains some lovely characterization of the developmental defects associated with loss of either Rab11a, Rab11b or the double knockout, including high quality micrographs with rigorous quantification of the localization defects of the various markers. The mechanistic work linking Rab11a to Kif11 is much less convincing, and there is no evidence provided that the effects are direct rather than indirect. There is also a lack of appropriate controls to show specificity of the interactions. I therefore cannot recommend publication of this manuscript. Below I have outlined my concerns with the manuscript to help the authors in their studies.

Major Concerns:

1. In Figure 2B and C, the authors score the Ki-67+ or PH3 positive cells per crypt, but it should be done as a fraction of the total nuclei since the Rab11a knockouts cause changes in cell cycle dynamics as well as ultimately lead to apoptosis.
2. In discussion of Figure 2M, the authors conclude that the CldU+ cells that were shedded off were still in the cell cycle. I disagree. CldU+ incorporation just shows that a cell went through S phase at some point in time, not that it continues to cycle unless there is a continued incorporation of labeled nucleotide.
3. The proteomic analysis with Rab11a showed a very large number of interactors, most of which are likely to be indirect. The follow-up co-IP studies could also likewise be indirect. To show a direct interaction, the authors would need to include in vitro work showing direct interactions. Alternatively, if the authors could identify the key sites of interactions, then they could do knockdown/rescue studies in cell culture systems to show reciprocal interactions and reciprocal effects on the function of the proteins.
4. For the colocalization studies in Figure 3, they are not at all convincing that this is a direct interaction. The spindle is composed of nearly 1000 proteins, and it has been shown many times that vesicles are excluded from the main area of the spindle but can surround the spindle itself. The Rab11 staining is in the vicinity of Kif11, but they are by no means coincident.
5. The majority of interactions with kinesin motor proteins occur through binding sites in the tail domains. The stalks are often part of the coiled coil interaction domain. Additional controls are needed to show specificity of this interaction.
6. The localization data in figure S3 is not convincing. I can't even detect the spindle clearly in these images.
7. The Kif11 inhibitors form monopolar spindles, and they should be called as such rather than abnormal spindles.
8. I do not understand what the authors are trying to conclude about the co-IP experiments with the different Kif11 inhibitors. These are allosteric effectors of the motor domain, which should have no effect on the stalk, which is the proposed site of interaction. In addition, there are no load controls in the gel, making the quantification not appropriate interpretable. A more likely explanation is that Rab11 interactions with another proteins whose localization/function is disrupted in the monopolar spindles induced by Kif11 inhibition.
9. In 3F, it is impossible to draw conclusions about the localization in multi-color images. Quantification would also be necessary to more convincingly show this point.
10. In the discussion of Figure 5A and B, the authors conclude that Kif11 localization is abnormal. In monopolar spindles, the poles are in the center of the aster, which is where the poles are located- thus the Kif11 staining is exactly where it is expected to be.
11. Kif11 has been well-studied with very few studies identifying bonafide interacting proteins. Thus, it is disconcerting that the present study reports a very large interactome with Kif11. The authors cite two papers (Busch 2004 and Williams 2003) as evidence that they got Kif11 interactors. Busch 2004 is about Tea2p in pombe, but Cut7 is the functional Kif11 ortholog in pombe. Likewise, the Williams paper is about identification of zwilch, which is part of the mitotic checkpoint complex associated with kinetochores on the chromosomes- thus in a place that Kif11 is not. The Williams paper does not discuss interactions with Klp61F, which is the Drosophila Kif11 ortholog. Thus, I have serious reservations about the validity of this interactome.
12. Overall, I think a more likely explanation of the present findings is that loss of Rab11 causes pleiotropic defects, which impact spindle structure and thus in turn results in mitotic arrest and disruption of spindle associated factors.

Minor Concerns:

1. The citation about Kif11 forming homo-tetramers is incorrect. This was first shown by Kashina and Scholey.
2. In panel 3I, how can the molecular weight of the stalk fragment be smaller in the IP sample than in the load?

Referee #3:

Joseph et al. report the implication of the small GTPase RAB11 in controlling the mitotic spindle stability in dividing intestinal epithelial cells. The authors found a novel physical interaction of RAB11A and B with the kinesin KIF11. KIF11 inhibition promotes a phenotype similar to RAB11A + RAB11B double knockout (DKO cells). Cell-cycle arrest, apoptosis, and loss of differentiated epithelium were observed in DKO cells. The authors propose a new molecular mechanism involving RAB11-KIF11 interaction crucial for maintenance of tissue homeostasis. This is a potentially interesting manuscript, however there are several issues that must be addressed to make this paper suitable for EMBOR audience. Below are my comments and suggestions:

Major Comments:

1) The immunocytochemistry analysis is questionable (e.g., Fig. 1P; +TAM 1d vs. 2d, the Olfm4 signal is not consistent with the quantification shown in 1Q). Quantification "Grey scale intensity" for DAB reaction is not recommended (high variability), replace "grey scale intensity" by positive cells per crypt in Figs. 1N, 1Q, 2C, and 6M. Include western blots (as in Fig. 1B) and the corresponding quantification for Olfm4 and Ki67. The size of crypts in DKO animals is reduced, when a significant reduction in positive cells is observed (e.g., Figs. 1J, 2D) provide the ratio; number of positive cells/total cells per crypt.

2) Data regarding pHH3 in Fig. 2 is not convincing, the DKO crypt phenotype is not evident in Fig. 1D and the ECAD signal is not consistent to data shown in Fig. 1G and K. Show images at lower magnification (as in Fig. 1K or 2J) and include western blot analysis for pHH3.

3) Figure 3G shows a moderate colocalization (r= 0.6) of Rab11 and KIF11 in mitotic spindles of metaphase HEK293 cells. Include in the analysis a spindle pole marker (e.g., gamma-tubulin). Live-cell imaging data (Fig. 3K-M) must show selected frames from time-lapse sequences during interphase and mitosis progression to follow the location of Rab11a in WT, S25N, and S20V expressing cells.

4) A previous study (PMID: 24561039) reported that either Rab11-depletion of S25N expressing cells show disrupted astral microtubules arrays, increased spindle orientation angles respect the substratum, and mitosis delay in prometa/metaphase. Include in the analysis lateral x-z confocal sections (z-axis views) with spindle poles in focus or representative 3D views of fixed cells co-stained with a spindle pole marker and DAPI (Fig. 3K-M) to discard i) the presence of Rab11a m-Cherry at the spindle poles and ii) that cells with monopolar/abnormal spindles (Fig. 3L) correspond to cells with tilted or orthogonal spindle angles. In this sense, the authors must provide better imaging evidence (including a marker for spindle pole), further analysis for the spindle phenotype (including DKO cells with monopolar/abnormal spindle and cells with bipolar tilted spindles) and protein (Kif11, Clip1, Ccar1, and Ccnb1) distribution in intestinal epithelia cells of DKO mice (Figs. 5A-C, 5J-M, O, P).

5) Images for IP:KIF11 CLIP1 and BUB3 (Fig. 5H) are pixelated, seem to be manipulated. Replace.

6) Quantifications shown in Figs. 5K and P (DKO) are not consistent with the levels of red:green overlap shown in the corresponding images; CLIP1:Kif11 colocalization at the mitotic spindle is similar in WT and DKO (Fig. 5J). The CLIP1:Kif11 colocalization in Fig. 5J is clearly higher than Ccnb1:Kif11 in Fig. 5O but similar Correlation Coefficient averages are shown in graphs. Authors must improve the colocalization analysis and provide detailed information regarding the criteria used for quantification in methods. Split channels in Figs. 3G, 3K-M, 4F, 5J, L, O, and s. Fig. 3C.

Minor issues:

-Figure 1; include a color scale bar in O
-Figure 2; be consistent with the type of graphs used, C or E. Include labels for cycle stages in F or fuse panels F-H
-Figure 3; Include a representative image of a cell with tripolar/multipolar spindles
-Figure 4; include the % of DMSO in control (vol/vol) (Methods). Mention H in main text
-Figure 5; in A, WT and DKO images seem to be manipulated (especially in the case the KIF11 signal histogram equalization), replace DKO. Images in C must be replaced, is hard to distinguish the metaphase plate in WT or identify Kif11 concentrated at the spindle pole in DKO cells. Point out CLIP1, ZW10, and Bub3b in G
-S. Figure 1; double check labeling in D
-S. Figures 3C-H; show single cells and DNA staining. A mixed phenotype is shown in S20V expressing cells (WT-like and diffuse cytosolic pattern in bottom cells). Quantify patterns.
-S. Figure 4. Include a mention to A in main text
-Mean and SD or SEM must be visible in graphs (Figs. 1H, J, L, N, Figs. 2C, E, Fig. 4G, and Fig. 6P)
-Include the P values in the Figure legends
-Include scale bars in all microscopy images shown in Figs. 1, 3, 4, 5, 6 and S. Figs. 1, 3, 4
-Supplementary Table; exclude DAPI and Vector Red

Point-by-point Response:

Referee #1

This manuscript describes the effects of knockout of both Rab11a and Rab11b in the mouse intestine. This work is novel because the ability to knock out both forms of Rab11 in vivo has been impossible due to embryonic lethality. Even so, double knockout of these proteins is rapidly lethal, indicating both their redundancy and importance. This is a comprehensive study that analyzes the effects on intestinal architecture, as well as effects on the intestinal epithelium itself. The impacts of Rab11 knockout are robust, with villus blunting, changes in enterocyte gene expression, changes in secretory lineages, and effect on stem cells. Strikingly, there is a substantial defect in the cell cycle, which is reflected in aberrant bipolar spindle assembly and arrested cell cycle. Co-immunoprecipitation finds interaction of Rab11 with multiple proteins known to be involved in cell division, common to both Rab11a and Rab11b, with Kif11 being ranked highest. Further experiments defined the importance of this interaction for normal spindle pole assembly and subsequent successful mitosis. Overall, this study that strongly links the cell autonomous effects of Rab11 knockout on intestinal epithelial proliferation through interaction with Kif11.
Minor concerns:
Figure 1A. All of the colors in the legend are not discernible on the figure. While one can infer that the blue and orange are under the black line, it would be preferable to represent them all.

Author Response: Fixed.

Figure 1P. The image quality in this panel is very low.

Author Response: Replaced with high quality images throughout the revised figures.

Figure 2C. While the text states that at 2d in the DKO there is a difference in Ki67 labeling, there is no statistical analysis of this time point.

Author Response: Quantifying ratio of Ki67+ epithelial cells over total epithelial cells suggested an increase at 2d (new Fig. 2C).

Figure 2F is redundant with 2G, H.

Author Response: Removed redundant panel.

In general, the fluorescent images are presented as a single merged panel. It would be useful to have the split channels together with the merged images to allow the reader to assess colocalization.

Author Response: This helpful suggestion is addressed by adding split channels to supplemental figures.

Figure 6O. The propridium iodide staining is relatively uninterpretable. It is clear that there is more, but assigning the staining to any population is difficult. 2D enteroids would be better suited to this experiment.

Author Response: Appreciate this helpful comment. We added a new figure (Fig. 6) and Fig. 7A-B to demonstrate the enteroid phenotypes, from various aspects. New experiments included staining and

quantification for cleaved caspase 3, pHH3, Kif11, histological assessment of the phenotype, as well as differentiation marker staining.

Referee #2

In the present manuscript the authors report a new role for Rab11 in bipolar spindle formation in mouse epithelia cell division, which they propose is mediated by the Kinesin-5 family member, Kif11. Loss of both Rab11 isoforms (a and b) causes cell cycle defects leading to apoptosis, which ultimately causes a loss of differentiated epithelial cells. They postulate that this is a novel mechanism for cell division and tissue homeostasis.

Author response: In revised paper, we added various new results to strengthen the major points that loss of Rab11a and Rab11b led to spindle defects, mitotic arrest, and apoptosis. In revised paper, we made it clear that loss of Rab11 affects the spindle function on a global scale, rather than being mediated exclusively through Kif11. We made it clear that Kif11 is just part of the program. We do not want to overstate that the observed cell division defect is mediated by Kif11. We think that Rab11a and Rab11b redundantly regulate tissue progenitor cell and stem cell renewal in vivo is novel.

The manuscript contains some lovely characterization of the developmental defects associated with loss of either Rab11a, Rab11b or the double knockout, including high quality micrographs with rigorous quantification of the localization defects of the various markers. The mechanistic work linking Rab11a to Kif11 is much less convincing, and there is no evidence provided that the effects are direct rather than indirect. There is also a lack of appropriate controls to show specificity of the interactions. I therefore cannot recommend publication of this manuscript. Below I have outlined my concerns with the manuscript to help the authors in their studies.

Author response: The impact of loss of Rab11 on cell division and tissue homeostasis are further strengthened by new enteroid experiments and in vivo results. In addition, we have performed experiments to try to address all the critiques below that were raised about the link between Rab11a and Kif11. Results from these new experiments helped clarifying direct versus indirect interactions. According to these experiments, we modified the narratives and provided substantial literature reviews for interpreting our observations.

Major Concerns:

1. In Figure 2B and C, the authors score the Ki-67+ or PH3 positive cells per crypt, but it should be done as a fraction of the total nuclei since the Rab11a knockouts cause changes in cell cycle dynamics as well as ultimately lead to apoptosis.

Author response: Now these proliferation markers are quantified as ratio of total IECs per crypt-villus axis (new Fig. 2C and Fig. 2I).

2. In discussion of Figure 2M, the authors conclude that the CldU+ cells that were shedded off were still in the cell cycle. I disagree. CldU+ incorporation just shows that a cell went through S phase at some

point in time, not that it continues to cycle unless there is a continued incorporation of labeled nucleotide.

Author response: We agree with reviewer. We meant that the shedding cells went through S phase and were undergoing cell death. This was consistent with cell-cycle arrest.

3. The proteomic analysis with Rab11a showed a very large number of interactors, most of which are likely to be indirect. The follow-up co-IP studies could also likewise be indirect. To show a direct interaction, the authors would need to include in vitro work showing direct interactions. Alternatively, if the authors could identify the key sites of interactions, then they could do knockdown/rescue studies in cell culture systems to show reciprocal interactions and reciprocal effects on the function of the proteins.

Author response: We agree. Proteomic experiments performed by co-IP methods are expected to precipitate protein complexes and some targets do not directly bind to our protein of interest. We have spent substantial time performing in vitro binding assays (S. Fig. 6B-C). We tested the interaction in buffer by using two approaches. We used in vitro transcription and translation (TnT) system to produce recombinant Kif11 fragments, and tested their interaction with purified recombinant Rab11 proteins. We also performed in vitro binding assays using full length KIF11. We repeated these in vitro binding assays many times, but did not found Kif11-Rab11 association, suggesting that the intracellular association was mediated through spindle associated protein complexes. We provided potential candidates, such as tubulin, dynactin, and FIP proteins that may mediate the association. Within the cells, we performed additional experiments to further test how depolymerizing spindle microtubules or inhibiting Kif11 motor function impact their associations. These experiments helped us to better interpret the observed biochemical and phenotypical results related to spindle defects.

4. For the colocalization studies in Figure 3, they are not at all convincing that this is a direct interaction. The spindle is composed of nearly 1000 proteins, and it has been shown many times that vesicles are excluded from the main area of the spindle but can surround the spindle itself. The Rab11 staining is in the vicinity of Kif11, but they are by no means coincident.

Author response: As requested below by reviewers, we performed new colocalization analysis using spindle pole markers pericentrin and showed that a fraction of Rab11 localized to the pericentriolar region (Fig. 4G), as well as mitotic spindle (S. Fig. 3C, 6A). Our observations confirmed previous reports that Rab11 and recycling endosome localize to the mitotic spindle and the poles (Ai *et al*, 2009; Zhang *et al*, 2008) (Hehnly & Doxsey, 2014), studies cited in original submission. Our study provided new insights by demonstrating the in vivo functional significance of such regulation, and the functional redundancy of two Rab11 isoforms in regulating spindle function. We now agree with the reviewer that Rab11 may complex with intermediate spindle-associated proteins that associate with Kif11. Rab11-interacting proteins, such as Rab11FIP3 (localizes to mitotic spindles), along with CLIP1 are present in microtubule organizing center. CLIP1 links recycling endosome to microtubules (Pierre *et al*, 1992). The co-precipitation of Rab11 and Kif11 is likely to represent an event of multi-protein complex assembled at the mitotic spindle. We provided substantial literature review and discussion about these results.

5. The majority of interactions with kinesin motor proteins occur through binding sites in the tail domains. The stalks are often part of the coiled coil interaction domain. Additional controls are needed to show specificity of this interaction.

Author response: Agree. Under the conditions tested in our in vitro binding assays, the association between Kif11 and Rab11 was likely mediated through spindle or other spindle-associated proteins, such as dynactin, CLIP1 or FIP proteins. We provided extensive discussion about the coiled coil domain in Kif11 stalk region. Kif11 stalk is directly phosphorylated by the Xenopus laevis aurora-related protein kinase (Giet et al, 1999). A direct association between KIF11 and dynactin [p150(Glued)] was previously isolated in yeast two-hybrid study using the stalk-tail region (420-811 aa.) as a bait (Blangy et al, 1997). Coincidentally, dynactin also directs Rab11 recycling endosomes to pericentrosomal region and spindle (Ai et al., 2009). Rab3-GEP directly binds to the stalk domain of KIF1Bbeta and KIF1A, to transport synaptic vesicles (Niwa et al, 2008). Almost all Rab11 FIPs use their conserved C-terminal coiled coil domain to bind Rab11 (Hales et al, 2001). Resolved complex structures of Rab11–FIP2, Rab11–FIP3, Rab14–FIP1 and Rab25–FIP2 suggested that all of the Rab11 binding domains contain a central parallel coiled coil (Kearney & Khan, 2020; Lall et al, 2015; Wei et al, 2006). FIP3 localizes to mitotic spindle (Hehnly & Doxsey, 2014), forms homo-oligomers, and is essential for cytokinesis (Eathiraj et al, 2006). FIP3's Rab11 binding domain contains a coiled coil homodimer with two symmetric interfaces binding to two Rab11 molecules (Shiba et al, 2006). The C- and N-terminal domains of the homodimeric CLIP1 are also connected by a long coiled coil (Pierre et al., 1992). We discussed these studies to suggest that the coiled coil motif of KIF11 stalk region may interface with various Rab11-interacting partners in a spindle microtubule associated complex. Although we did not detect KIF11-RAB11 binding in buffer under the tested conditions, overexpressing KIF11 stalk in cells caused spindle defects in our new experiments (Fig. 5D, S. Fig. 6A). In addition, The direct phosphorylation at threonine 937 in KIF11 C-terminal tail by Cdk1-Cyclin B strongly increased its binding to microtubules in vitro (Cahu et al, 2008). Interestingly, we observed a strong association between KIF11 and Cyclin B in Rab11-depleted cells, suggesting that Rab11 endosome regulates KIF11 interactions with other mitotic regulators.

6. The localization data in figure S3 is not convincing. I can't even detect the spindle clearly in these images.

Author response: we have performed new experiments and included new images throughout the revised figure panels to demonstrate localization of Rab11 and Kif11 relative to spindle and spindle poles (labeled by gamma-tubulin or pericentrin). Fig. 4G, 4L-M, S. Fig. 3C, S. Fig. 6A.

7. The Kif11 inhibitors form monopolar spindles, and they should be called as such rather than abnormal spindles.

Author response: corrected.

8. I do not understand what the authors are trying to conclude about the co-IP experiments with the different Kif11 inhibitors. These are allosteric effectors of the motor domain, which should have no effect on the stalk, which is the proposed site of interaction. In addition, there are no load controls in the gel, making the quantification not appropriate interpretable. A more likely explanation is that Rab11 interactions with another proteins whose localization/function is disrupted in the monopolar spindles induced by Kif11 inhibition.

Author response: Based on our new in vitro binding results, we agree with reviewer's point "A more likely explanation is that Rab11 interactions with another proteins whose localization/function is disrupted in the monopolar spindles induced by Kif11 inhibition."

Kif11 chemical inhibitors are valuable pharmacological tools, and our purpose of using them was to leverage their ability of blocking the motor function thereby testing the intracellular association with Rab11. The data suggested that these inhibitors decreased but not eliminate their association. As these drugs can change the abundance of total cellular Kif11, our quantification was performed for the ratio of co-IP Kif11 against the total cellular Kif11 (as the input) of same amount of cell lysates. Now, we also included the blot for Rab11a in Fig. 7F to demonstrate the even loading.

9. In 3F, it is impossible to draw conclusions about the localization in multi-color images. Quantification would also be necessary to more convincingly show this point.

Author response: These images have been replaced with new ones with split and merged channels (Fig. 4G) and Z-stack 3D projections (S. Fig. 3C). Pearson's correlations of Rab11 and Kif11 at spindle poles defined by pericentrin are now reported in the results.

10. In the discussion of Figure 5A and B, the authors conclude that Kif11 localization is abnormal. In monopolar spindles, the poles are in the center of the aster, which is where the poles are located- thus the Kif11 staining is exactly where it is expected to be.

Author response: This point is well taken. We have revised the descriptions according to reviewer's suggestion.

11. Kif11 has been well-studied with very few studies identifying bonafide interacting proteins. Thus, it is disconcerting that the present study reports a very large interactome with Kif11. The authors cite two papers (Busch 2004 and Williams 2003) as evidence that they got Kif11 interactors. Busch 2004 is about Tea2p in pombe, but Cut7 is the functional Kif11 ortholog in pombe. Likewise, the Williams paper is about identification of zwilch, which is part of the mitotic checkpoint complex associated with kinetochores on the chromosomes- thus in a place that Kif11 is not. The Williams paper does not discuss interactions with Klp61F, which is the Drosophila Kif11 ortholog. Thus, I have serious reservations about the validity of this interactome.

Author response: we apologize for the confusion. The citations of (Busch 2004 and Williams 2003) were not used to describe Kif11, but to refer to Clip1 and ZW10. We have revised description, interpretation, and literature citation related to this piece of data. We also performed extra experiments to support the validity of this proteomic experiment. First, we are making the point clear that the KIF11 pull down assay was not purposed for identifying direct KIF11-binding partners. We used it to obtain an untargeted profiling of the protein network that came down with KIF11, directly or indirectly. As KIF11 crosslinks the spindle microtubules, we now stated in the revised paper that, the antibody-mediated KIF11 immunoprecipitation from Caco2 cell lysates used in our experiments, were likely to pull down spindle-associated large protein complexes, within which many proteins may not directly interact with KIF11. As KIF11 binds mitotic spindle microtubules that attach to the kinetochores, it was not entirely surprising to detect components of these mitotic machineries in our proteomics.
Second, the antibody that used to IP KIF11 was validated by us using recombinant KIF11 protein (S. Fig. 6C).
Third, we used the proteomic results of negative control IP reactions, where no KIF11 antibody was added in the pull-down assay. Thus, we have subtracted the non-specific background from the KIF11 proteomics. These have now been described in the revised methods.

12. Overall, I think a more likely explanation of the present findings is that loss of Rab11 causes pleiotropic defects, which impact spindle structure and thus in turn results in mitotic arrest and disruption of spindle associated factors.

Author response: we agree with the review's interpretation of the overall phenotype, and revised our discussion accordingly.

Minor Concerns:

1. The citation about Kif11 forming homo-tetramers is incorrect. This was first shown by Kashina and Scholey.

Author response: corrected the citation.

2. In panel 3I, how can the molecular weight of the stalk fragment be smaller in the IP sample than in the load?

Author response: Fixed.

Referee #3

Joseph et al. report the implication of the small GTPase RAB11 in controlling the mitotic spindle stability in dividing intestinal epithelial cells. The authors found a novel physical interaction of RAB11A and B with the kinesin KIF11. KIF11 inhibition promotes a phenotype similar to RAB11A + RAB11B double knockout (DKO cells). Cell-cycle arrest, apoptosis, and loss of differentiated epithelium were observed in DKO cells. The authors propose a new molecular mechanism involving RAB11-KIF11 interaction crucial for maintenance of tissue homeostasis. This is a potentially interesting manuscript, however there are several issues that must be addressed to make this paper suitable for EMBOR audience. Below are my comments and suggestions:

Major Comments:

1) The immunocytochemistry analysis is questionable (e.g., Fig. 1P; +TAM 1d vs. 2d, the Olfm4 signal is not consistent with the quantification shown in 1Q). Quantification "Grey scale intensity" for DAB reaction is not recommended (high variability), replace "grey scale intensity" by positive cells per crypt in Figs. 1N, 1Q, 2C, and 6M. Include western blots (as in Fig. 1B) and the corresponding quantification for Olfm4 and Ki67. The size of crypts in DKO animals is reduced, when a significant reduction in positive cells is observed (e.g., Figs. 1J, 2D) provide the ratio; number of positive cells/total cells per crypt.

Author response: according to the reviewer's above comments, we have performed the suggested immunocytochemistry analysis, replaced "grey scale" quantification with positive cells per crypt or per crypt-villus axis, and plotted ratio of positive cells against total IECs. These changes were made throughout related figure panels. New Western blots for Olfm4 have been added (Fig. 2G). We have technical difficulty to resolve Ki67, a protein of 358 kDa, from the tissue lysates. We were able to complete pHH3 Western blot as requested below.

2) Data regarding pHH3 in Fig. 2 is not convincing, the DKO crypt phenotype is not evident in Fig. 1D and the ECAD signal is not consistent to data shown in Fig. 1G and K. Show images at lower magnification (as in Fig. 1K or 2J) and include western blot analysis for pHH3.

Author response: New images of pHH3 staining and Western blot of pHH3 have been included (Fig. 2G-I). We have also included additional lower magnifications (Fig. 1F, Fig. 2B, S. Fig. 2B) to demonstrate the crypt phenotype. Additional panels were added for Fig. 3D, 3F to demonstrate the defects of cell cycle.

3) Figure 3G shows a moderate colocalization (r= 0.6) of Rab11 and KIF11 in mitotic spindles of metaphase HEK293 cells. Include in the analysis a spindle pole marker (e.g., gamma-tubulin). Live-cell imaging data (Fig. 3K-M) must show selected frames from time-lapse sequences during interphase and mitosis progression to follow the location of Rab11a in WT, S25N, and S20V expressing cells.

Author response: We have now included spindle pole markers (pericentrin and gamma-tubulin) in Fig. 4G, 4L, 4M, S. Fig. 3B-C). We have included time lapse frames for Rab11a WT, S25N and S20V of live cells (S. Fig. 4A). Based on spindle pole markers, new Pearson's correlation values are reported in results.

4) A previous study (PMID: 24561039) reported that either Rab11-depletion of S25N expressing cells show disrupted astral microtubules arrays, increased spindle orientation angles respect the substratum, and mitosis delay in prometa/metaphase. Include in the analysis lateral x-z confocal sections (z-axis views) with spindle poles in focus or representative 3D views of fixed cells co-stained with a spindle pole marker and DAPI (Fig. 3K-M) to discard i) the presence of Rab11a m-Cherry at the spindle poles and ii) that cells with monopolar/abnormal spindles (Fig. 3L) correspond to cells with tilted or orthogonal spindle angles. In this sense, the authors must provide better imaging evidence (including a marker for spindle pole), further analysis for the spindle phenotype (including DKO cells with monopolar/abnormal spindle and cells with bipolar tilted spindles) and protein (Kif11, Clip1, Ccar1, and Ccnb1) distribution in intestinal epithelia cells of DKO mice (Figs. 5A-C, 5J-M, O, P).

Author response: we have now provided 3D views of Rab11 WT, S25N, and S20V, along with a spindle pole marker (pericentrin) in focus, as suggested by the reviewer (S. Fig. 3C). Additional images were added for DKO cells with monopolar spindle, abnormal spindle, and bipolar tilted spindles (Fig. 4G, 4I, 4J, 4M, S. Fig. 5A-B). We also included images of tripolar and multipolar spindles induced by Rab11 S25N overexpression (S. Fig. 3B). We discussed our results in light of (PMID: 24561039). Technically, the antibody species availability prevented us from simultaneously staining for Kif11, Rab11, spindle pole together with Clip1 or Ccar1, at the same time.

5) Images for IP:KIF11 CLIP1 and BUB3 (Fig. 5H) are pixelated, seem to be manipulated. Replace.

Author response: replaced, Fig. 5N.

6) Quantifications shown in Figs. 5K and P (DKO) are not consistent with the levels of red:green overlap shown in the corresponding images; CLIP1:Kif11 colocalization at the mitotic spindle is similar in WT and DKO (Fig. 5J). The CLIP1:Kif11 colocalization in Fig. 5J is clearly higher than Ccnb1:Kif11 in Fig. 5O but similar Correlation Coefficient averages are shown in graphs. Authors must improve the colocalization analysis and provide detailed information regarding the criteria used for quantification in methods. Split channels in Figs. 3G, 3K-M, 4F, 5J, L, O, and s. Fig. 3C.

Author response: Split channels are provided for Clip1:Kif11 (Fig. 5N). We double checked our correlation analysis. Criteria used for Pearson's correlation and select of region of interest (in most case, the spindle or spindle pole) are detailed in methods.

Minor issues:

-Figure 1; include a color scale bar in O

Author response: included.

-Figure 2; be consistent with the type of graphs used, C or E. Include labels for cycle stages in F or fuse panels F-H

Author response: we now made all type of graphs consistent. Panel F is removed.

-Figure 3; Include a representative image of a cell with tripolar/multipolar spindles

Author response: we included images of tripolar and multipolar spindles (S. Fig. 3B).

-Figure 4; include the % of DMSO in control (vol/vol) (Methods). Mention H in main text

Author response: included. The original schematic diagram H is removed.

-Figure 5; in A, WT and DKO images seem to be manipulated (especially in the case the KIF11 signal histogram equalization), replace DKO. Images in C must be replaced, is hard to distinguish the metaphase plate in WT or identify Kif11 concentrated at the spindle pole in DKO cells. Point out CLIP1, ZW10, and Bub3b in G

Author response: we have provided a variety of images in current main Figure 4 and S. Fig. 5 to demonstrate how Kif11 spindle appear in WT and DKO tissues. Likewise, we replaced the original panel C with new enteroid images of Kif11 spindles in Fig. 6 to provide a variety of examples of spindle morphology in WT and mutant tissues. Panel G has been moved to Supplement.

-S. Figure 1; double check labeling in D

Author response: checked.

-S. Figures 3C-H; show single cells and DNA staining. A mixed phenotype is shown in S20V expressing cells (WT-like and diffuse cytosolic pattern in bottom cells). Quantify patterns.

Author response: we now described the mixed phenotype in result.

-S. Figure 4. Include a mention to A in main text

Author response: included now.

-Mean and SD or SEM must be visible in graphs (Figs. 1H, J, L, N, Figs. 2C, E, Fig. 4G, and Fig. 6P)

Author response: Mean and SEM are clearly plotted in all graphs.

-Include the P values in the Figure legends

Author response: we designated the p-value representation of individual asterisks.

-Include scale bars in all microscopy images shown in Figs. 1, 3, 4, 5, 6 and S. Figs. 1, 3, 4

Author response: scale bars are added to all images.

-Supplementary Table; exclude DAPI and Vector Red

Author response: done.

References for this document:

Ai E, Poole DS, Skop AR (2009) RACK-1 directs dynactin-dependent RAB-11 endosomal recycling during mitosis in Caenorhabditis elegans. *Mol Biol Cell* 20: 1629-1638

Blangy A, Arnaud L, Nigg EA (1997) Phosphorylation by p34cdc2 protein kinase regulates binding of the kinesin-related motor HsEg5 to the dynactin subunit p150. *J Biol Chem* 272: 19418-19424

Cahu J, Olichon A, Hentrich C, Schek H, Drinjakovic J, Zhang C, Doherty-Kirby A, Lajoie G, Surrey T (2008) Phosphorylation by Cdk1 increases the binding of Eg5 to microtubules in vitro and in Xenopus egg extract spindles. *PLoS One* 3: e3936

Eathiraj S, Mishra A, Prekeris R, Lambright DG (2006) Structural basis for Rab11-mediated recruitment of FIP3 to recycling endosomes. *Journal of molecular biology* 364: 121-135

Giet R, Uzbekov R, Cubizolles F, Le Guellec K, Prigent C (1999) The Xenopus laevis aurora-related protein kinase pEg2 associates with and phosphorylates the kinesin-related protein XlEg5. *J Biol Chem* 274: 15005-15013

Hales CM, Griner R, Hobdy-Henderson KC, Dorn MC, Hardy D, Kumar R, Navarre J, Chan EK, Lapierre LA, Goldenring JR (2001) Identification and characterization of a family of Rab11-interacting proteins. *J Biol Chem* 276: 39067-39075

Hehnly H, Doxsey S (2014) Rab11 endosomes contribute to mitotic spindle organization and orientation. *Dev Cell* 28: 497-507

Kearney AM, Khan AR (2020) Crystal structure of the Rab-binding domain of Rab11 family-interacting protein 2. *Acta Crystallogr F Struct Biol Commun* 76: 357-363

Lall P, Lindsay AJ, Hanscom S, Kecman T, Taglauer ES, McVeigh UM, Franklin E, McCaffrey MW, Khan AR (2015) Structure-Function Analyses of the Interactions between Rab11 and Rab14 Small GTPases with Their Shared Effector Rab Coupling Protein (RCP). *J Biol Chem* 290: 18817-18832

Niwa S, Tanaka Y, Hirokawa N (2008) KIF1Bbeta- and KIF1A-mediated axonal transport of presynaptic regulator Rab3 occurs in a GTP-dependent manner through DENN/MADD. *Nat Cell Biol* 10: 1269-1279

Pierre P, Scheel J, Rickard JE, Kreis TE (1992) CLIP-170 links endocytic vesicles to microtubules. *Cell* 70: 887-900
Shiba T, Koga H, Shin HW, Kawasaki M, Kato R, Nakayama K, Wakatsuki S (2006) Structural basis for Rab11-dependent membrane recruitment of a family of Rab11-interacting protein 3 (FIP3)/Arfophilin-1. *Proc Natl Acad Sci U S A* 103: 15416-15421
Wei J, Fain S, Harrison C, Feig LA, Baleja JD (2006) Molecular dissection of Rab11 binding from coiled-coil formation in the Rab11-FIP2 C-terminal domain. *Biochemistry* 45: 6826-6834
Zhang H, Squirrell JM, White JG (2008) RAB-11 permissively regulates spindle alignment by modulating metaphase microtubule dynamics in Caenorhabditis elegans early embryos. *Mol Biol Cell* 19: 2553-2565

Dear Nan,

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of reports from the three referees that were asked to re-evaluate your study. Please find their comments below.

As you will see, referees #1 and #3 find the revised study substantially improved and their previous concerns addressed, and they now support publication in EMBO reports. Referee #2, on the other hand, points out that the work is now mostly descriptive and the level of provided molecular insight limited, although they also recognize that the comments and concerns of the referees were addressed. I have discussed your revision and the referees' comments with the other members of our editorial team, and I am glad to inform you that we can offer publication of your manuscript in our journal despite the incomplete molecular understanding of the underlying process.

From the editorial side, there are also a few things that we need from you before we can proceed with acceptance of the manuscript:

- Your Figure legends have been inspected by our data editors for completeness and accuracy. Please see the required changes in the attached Word file and address all comments in a revised manuscript (with tracked changes).

- Please provide up to 5 keywords in your revised manuscript.

- Please make sure that the datasets you have deposited in external repositories are publicly available at the time of publication (you can therefore remove reviewer tokens from the Data availability statement in the manuscript).

- You are kindly requested to consider both actual and perceived competing interests and include a relevant statement under the heading "Disclosure and competing interests statement". Please review our policy here: https://www.embopress.org/competing-interests.

- Please include your funding information in the revised manuscript (in a section called "Acknowledgements") and note that it should match the information entered into our online submission platform.

- We noticed that callouts of Fig. S2D and S3A are missing; please make sure that all Figure panels are called out (in alphabetical order) in your revised manuscript.

- Your supplementary table (please see also next point regarding supplementary materials) should also be called out in the manuscript.

- We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures (following the heading "Expanded View Figure Legends"). For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called "Appendix", which should start with a short Table of Contents (including page numbers). Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. Your supplementary table should be labeled and referred to as "Table EV1" (its legend can be provided either in a separate tab in case of Excel files or, alternatively, as a separate text file zipped together with the Table file. Alternatively, you could include the Table in the Appendix; in that case, it should be labeled and referred to in the main text as "Appendix Table S1". You can find more detailed instructions regarding Expanded View items and the Appendix here:
<https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Please note that EMBO press papers are accompanied online by:
A) a short (1-2 sentences) summary of the findings and their significance,
B) 2-4 short bullet points highlighting the key results, and
C) a synopsis image that is exactly 550 pixels wide and 300-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that the text needs to be readable at the final size.
Please upload this information along with your revised manuscript (the text for A and B should be provided in a separate Word file).

- We noticed that source data for Fig. 2F are missing, please include this dataset in your re-submission.

- Please follow this order of sections in your revised manuscript: Title page, Abstract, Keywords, Introduction, Results, Discussion, Materials and Methods, Data availability, Acknowledgements, Disclosure and competing interests statement, References, Figure legends, Expanded View Figure legends.

Please also note that as part of the EMBO publications' Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You can opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We look forward to seeing a final version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

This revised manuscript details the effects of Rab11a/b double knockout in orientation of the spindle in the intestinal epithelium. They find that Rab11 DKO impacts spindle orientation, which subsequently affects proliferation and apoptosis. Further, have established that the impact of Rab11 DKO is not due to a direct interaction with Kif11 but rather is likely mediated through a complex associated with the spindle. The authors have addressed my concerns regarding the manuscript through the inclusion of substantially improved figures.

Referee #2:

This is a revised version of a manuscript that previously concluded that RAB11A and RAB11B act through Kif11 to modulate spindle function. After addressing the reviewer comments, the entire story about the molecular mechanism has changed because the requested experiments showed that the interactions between RAB11A and RAB11B and Kif11 are indirect, and the authors now provide no real insight into how the RABs cause spindle defects. There is a lot of hand-waving in the discussion about previous work, but at the end of the day, this paper is mainly a nice descriptive report of RAB11A and RAB11B double knockout cells. It is more suitable for a specialized journal as it does not provide the molecular insights usually found in papers in EMBO Reports.

Referee #3:

The authors have reasonably addressed the issues raised in my previous review.

Response to editorial comments:

From the editorial side, there are also a few things that we need from you before we can proceed with acceptance of the manuscript:

- Your Figure legends have been inspected by our data editors for completeness and accuracy. Please see the required changes in the attached Word file and address all comments in a revised manuscript (with tracked changes).

Addressed with track changes.

- Please provide up to 5 keywords in your revised manuscript.

Provided.

- Please make sure that the datasets you have deposited in external repositories are publicly available at the time of publication (you can therefore remove reviewer tokens from the Data availability statement in the manuscript).

Database have become public.

- You are kindly requested to consider both actual and perceived competing interests and include a relevant statement under the heading "Disclosure and competing interests statement". Please review our policy here: https://www.embopress.org/competing-interests.

Disclosure statement added.

- Please include your funding information in the revised manuscript (in a section called "Acknowledgements") and note that it should match the information entered into our online submission platform.

Funding information added.

- We noticed that callouts of Fig. S2D and S3A are missing; please make sure that all Figure panels are called out (in alphabetical order) in your revised manuscript.

Call-out added.

- Your supplementary table (please see also next point regarding supplementary materials) should also be called out in the manuscript.

The table is now called out as Appendix Table S1.

- We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures (following the heading "Expanded View Figure Legends"). For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called "Appendix", which should start with a short Table of Contents (including page numbers). Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. Your supplementary table should be labeled and referred to as "Table EV1" (its legend can be provided either in a separate tab in case of Excel files or, alternatively, as a separate text file zipped together with the Table file. Alternatively, you could include the Table in the Appendix; in that case, it should be labeled and referred to in the main text as "Appendix Table S1". You can find more detailed instructions regarding Expanded View items and the Appendix here:
<https://www.embopress.org/page/journal/14693178/authorguide#expandedview>;

Have made according changes to show five Expended View figures and 2 Appendix figures.

- Please note that EMBO press papers are accompanied online by:
A) a short (1-2 sentences) summary of the findings and their significance,
B) 2-4 short bullet points highlighting the key results, and
C) a synopsis image that is exactly 550 pixels wide and 300-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that the text needs to be readable at the final size.
Please upload this information along with your revised manuscript (the text for A and B should be provided in a separate Word file).

We uploaded a word file that has narratives for the Summary (A), bullet points (B) and legend for synopsis image (C)

- We noticed that source data for Fig. 2F are missing, please include this dataset in your re-submission.

Source data for Fig. 2F added.

- Please follow this order of sections in your revised manuscript: Title page, Abstract, Keywords, Introduction, Results, Discussion, Materials and Methods, Data availability, Acknowledgements, Disclosure and competing interests statement, References, Figure legends, Expanded View Figure legends.

The paper is reorganized to follow the sequence.

Please also note that as part of the EMBO publications' Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You can opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Dear Dr. Gao

Thank you for the submission of your revised manuscript to our offices. Having gone through your manuscript could you please fix/improve a few remaining issues:

1. Please improve your title: it should be as specific and accurate as possible regarding the organism/tissue/cell type (and note that the revised title should be up to 100 characters including spaces).

2. Please improve your abstract: it should be written in present tense and not exceed 175 words.

3. Please provide the number of replicates in the legend of Fig. 5D.

4. The length of each scale bar in microscopy images should be defined in the legend AND be removed from the bar itself.

5. The synopsis image should be understandable by our general audience without the need for a legend. Please add the necessary annotation to the image (and make sure that all text will be readable at the final image size).

I look forward to seeing a new revised version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Ioannis Papaioannou, PhD
Editor
EMBO reports

The authors have addressed all minor editorial requests.

Dr. Nan Gao
Rutgers University
Biological Sciences
195 University Ave. Boyden Hall 206
Newark, NJ 07102
United States

Dear Nan,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

****

THINGS TO DO NOW:

Please note that you will be contacted by Wiley Author Services to complete licensing and payment information. The required 'Page Charges Authorization Form' is available here: https://www.embopress.org/pb-assets/embo-site/er_apc.pdf - please download and complete the form and return to embopressproduction@wiley.com

EMBO Press participates in many Publish and Read agreements that allow authors to publish Open Access with reduced/no publication charges. Check your eligibility: https://authorservices.wiley.com/author-resources/Journal-Authors/open-access/affiliation-policies-payments/index.html

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2022-56240V4 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

EMBO Press Author Checklist

Corresponding Author Name: Nan Gao	
Journal Submitted to: EMBO Report	
Manuscript Number: EMBOR-2022-56240	

USEFUL LINKS FOR COMPLETING THIS FORM
The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January 2022)
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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 χ2 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Mouse models in Methods

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Table included in Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Methods

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Methods

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Methods

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Methods
Include a statement about **blinding** even if no blinding was done.	Yes	Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Methods
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Methods, statistical analysis

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Yes	Methods
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	DATA AVAILABILITY SECTION
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	