Rab18 regulates focal adhesion dynamics by interacting with kinectin-1 at the endoplasmic reticulum

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September 7, 2018

Re: JCB manuscript #201809020

Prof. Cinzia Progida
University of Oslo
Blindernveien 31
Oslo 0371
Norway

Dear Prof. Progida,

Thank you for submitting your Article manuscript entitled "Rab18 regulates focal adhesion dynamics by recruiting kinectin-1 at the endoplasmic reticulum" to Journal of Cell Biology. As part of our normal reviewing procedure, your paper has been evaluated by at least two editors and an editorial statement is provided below. You will see that, in the consensus opinion of our editors, although we are interested in the concepts presented in this study, the manuscript is too preliminary for external review. We have thus decided not to subject your manuscript to a lengthy review process. We would be willing to consider a revised manuscript containing data addressing the detailed editorial comments below, assuming the novelty of the findings has not been compromised in the interim.

Because Journal of Cell Biology addresses a wide and diverse audience of cell biologists, we must give priority to manuscripts that provide a substantial advance of broad appeal to the cell biology community, even though many others also present interesting and important advances for researchers in a particular field.

I am sorry that our answer on this occasion is not more positive, and I hope that this outcome will not dissuade you from submitting other manuscripts to us in the future.

Thank you for your interest in Journal of Cell Biology.

With kind regards,

Jodi Nunnari
Editor-in-Chief
Journal of Cell Biology

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Editorial Statement:

In this study, the authors have provided an interesting description of a Rab regulating migration via ER-focal adhesions with the report that Rab18 interacts with the integral ER protein kinectin-1 and mediates ER transport to FAs for the regulation of FA dynamics during cell spreading. This study provides a potential mechanistic explanation for the previously described role of Rab18 in cell migration, which will certainly be appreciated by researchers in the field. However, given that kinectin-1 has a well-established role in ER transport for FAs, to appeal to the broad readership of
the Journal of Cell Biology, further mechanistic insight into the role of Rab18 in the regulation of
kinectin-1 and ER transport would be required. We would be happy to reconsider a suitably
developed and expanded manuscript that provides such novel insight into the mechanistic role of
Rab18 at ER-focal adhesions.
September 8, 2019

Re: JCB manuscript #201809020R-A

Prof. Cinzia Progida  
University of Oslo  
Blindernveien 31  
Oslo 0371  
Norway

Dear Prof. Progida,

Thank you for submitting your manuscript entitled "Rab18 regulates focal adhesion dynamics by interacting with kinectin-1 at the endoplasmic reticulum". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

Both reviewers are interested in the possibility that Rab18 controls cell migration but both recommend that a significant mechanistic extension is necessary to confirm and explain how Rab18 directly affects kinectin's functions in the ER and focal adhesions. In addition, the reviewers raise other, more specific technical issues with the data presented that should be addressed for resubmission. As the previous editorial decision recommended that "strong evidence of the mechanistic role of Rab18 in kinectin-1 function" would likely be necessary for JCB, if you wish to resubmit, we advise you to provide a detailed revision plan at an early stage to avoid spending time on experimental revisions that may not be sufficient.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.
Reviewer #1 (Comments to the Authors (Required)):

The authors present some interesting but preliminary findings on a potential role for Rab18 and kinectin in cell migration. This extends previous studies by other workers showing that the ER contributes to cell migration, and that, in part, this is mediated by the ER integral membrane protein kinectin. In this study the authors provide some evidence that Rab18 binds to kinectin. However, how this alters kinectin function is unclear. Kinectin is already associated with the ER, so it is unlikely to be membrane recruitment. Focal adhesion dynamics are altered, but a direct mechanistic link from Rab18 and kinectin is not established. Therefore, the effects could be indirect and relate to alter ER morphology and dynamics, functions already associated with Rab18 and kinectin.

The introduction section is poorly structured and does not make it particularly clear what ideas the authors are testing or what led them to investigate Rab18 and the role of the ER in cell migration. This is made worse since part of the introduction is in the first part of the results section. A major omission is the lack of introduction of published work on the role of the ER and kinectin in cell migration. In a number of instances, statements are gathered together and referenced as if all the citations support the points being made: "Indeed, Rab18 is also involved in the regulation of ER trafficking and structure as well as secretory granule transport (Dejgaard et al., 2008;
Gerondopoulos 136 et al., 2014; Vazquez-Martinez et al., 2007). These three publications make different claims. Dejgaard relates to ER-Golgi trafficking, Gerondopoulos relates to ER structure but not trafficking, and Vazquez-Martinez relates to secretory granules. This should be discussed and the information relevant for this study presented to the reader, not simply bundled together in this way.

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Modest changes in cell migration behavior are seen in Rab18 knockdown cells after 21h. This seems reflect a general slowing down in wound closure. Comparison with other Rabs isn't performed, and this effect appears modest to some other previous studies where much larger effects were observed with endocytic recycling Rabs such as Rab4 and Rab11. The main text states that the rate of wound closure was reduced by over 20%. However, the graph in Figure 1b shows a delay in closure, but after that the slope of the curves looks similar. Looking at the data, we can conclude that the rate of closure is similar, but the extent of closure at 21h is reduced by ~15%. Since the migration of individual cells has not been tracked to measure velocity, I am not sure that "rate" should be used here. Figure 1d and 1e are important, but supplemental data.

Figure 1a. What percentage of cells are transfected for the rescue with GFP-Rab18? Normally this is <50%, which would be expected to limit recovery. Can the authors show the GFP channel? Did the GFP-positive cells migrate more than those not rescued?

The authors use U2OS cells. Are the results obtained seen with other cell lines? MDA-MB-231 cells have been used in another study of the role of kinectin and ER in cell migration, so it would be valuable to look at Rab18 in those cells. This would also enable the authors to look in more detail are the behavior of cell protrusions, and whether knockdown of Rab18 results in the loss of preferential orientation towards a chemoattractant.

Figure 4h. Traction force should be in absolute units, not normalised. This hides variation in the control values. This is also an issue with the way data is presented in some other figures (see Figure 4e, 5f, 7b).

What role does the Rab18-kinectin interaction play in FA dynamics and cell migration? The referenced Ng & Zhang papers indicate that ER spreading and kinectin and linked to migration. To extend this, the authors need to produce stable cell lines expressing different kinectin mutants defective for Rab18 binding and test these in the various assays used in Figures 1-4 for Rab18. As already mentioned, examination of chemotaxis may also prove valuable.

Different ER markers should be compared to the kinectin staining in control and Rab18 depleted cells for Figure 5c. Sheet and tubule compartments such as CLIMP63 and reticulons should be examined. This also needs to be extended to the localization of mutant kinectin constructs within the ER (Figure 6).

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Corrections:
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2. Figure 6e. Spelling mistake. "length" not "leght".
3. Scale bars should be present for enlarged panels. This is not always the case. (Figure 5a, 5e, 7a)
4. Use proper gene names, so KNT1 (kinectin) not KNT-1. In some instances, KNT1 or KNT-1 is used and in other KNT only. Please edit for consistency.

Reviewer #2 (Comments to the Authors (Required)):

This paper from the Progida lab describes experiments aimed at elucidating a role for Rab18 in cell adhesion and migration and, more specifically, to link Rab18’s activities at the endoplasmic reticulum (ER) to focal adhesion turnover. The authors find that depletion of Rab18 compromises cell migration and adhesion, and that this is likely owing to decreased focal adhesion (FA) assembly and decreased FA turnover. The authors then turn their attention to how Rab18 depletion alters the distribution of the ER and how the dynamics of ER elements might relate to FA turnover. In particular, they find that Rab18 depletion reduces the contact of ER elements with vinculin-containing FAs. Finally, the authors show that Rab18 associates with the microtubular motor kinesin-1 via an interaction mediated by kinectin-1 - with Rab18 associating with kinectin via a region corresponding to the 201-445 stretch of amino acids of kinectin-1 - and that a mutant of kinectin-1 lacking residues 201-445, that is unable to bind to Rab18, does not restore contact of FAs with the ER.

General comment:
The finding that Rab18 influences cell adhesion and migration is of interest to the readership of JCB. Moreover, most of the work presented in this manuscript is of good quality and appears to be well-controlled and quantified. However, in terms of mechanistic insight the paper appears to be too preliminary for publication in JCB. I feel that the authors should provide more evidence for how Rab18 influences the dynamics of the ER and how this, in turn, alters FA dynamics. With this additional mechanistic insight, the paper would likely be suitable for JCB.

Specific comments:
1. The cell migration data presented in Fig. 1 are not convincing. Differences in wound closure can owe to a number of reasons, and a reduction in cell migration speed is only one of them. The authors need to analyse the influence of Rab18 on cell migration in more detail by using time-lapse microscopy followed by cell tracking. Furthermore, the authors need to link the various adhesive metrics that they have - cell spreading; FA assembly and disassembly; altered cell contractility - to the movement of cells. Indeed, it is not a given that reduced adhesion will lead to reduced cell migration. Very often, it’s the reverse because highly adherent cell can struggle to move across 2D surfaces. To me, it seems likely that, given their vestigial actin stress fibers, Rab18 depleted cells are not able to generate the traction forces necessary to translocate their cell bodies. Maybe, this could be investigated.
2. The adhesion/migration-related approaches that the authors choose to use throughout the paper seems a little inconsistent. For instance, why have they looked at FA assembly/disassembly and contractility in Fig 4., but not subsequently? Similarly, why do the authors measure the effect of depletion of kinectin-1 and/or expression of Rab18-binding deficient mutants of kinectin-1 on FA/ER contact sites, but not on FA turnover and/or cell migration?
3. As the authors mention, previous work on the role of kinectin and the ER in cell adhesion and migration analysed the dynamics of the ER with respect to cell protrusion and concluded that kinectin-mediated recruitment of the ER into extending lamellipodia facilitates the subsequent recruitment of FA proteins, such as β3 integrins and then vinculin (Zhang et al., 2010). Interestingly, one of the authors of this paper, Gareth Griffiths, is a departmental colleague of the authors of the present manuscript. Surely, if the authors believe that Rab18 is required for this process to occur,
then they should test it experimentally, and provide some mechanistic data as to why this is so.

4. The data in Fig. 3 are not convincing to this reviewer. I am not able to see evidence for a 'clear correlation between the two proteins, with both Rab18 and vinculin intensities increasing during FA assembly or decreasing during FA disassembly'. This experiment needs to be conducted in a way that is statistically meaningful.

5. I do not understand why the authors have chosen to perform the pillar experiment in Fig 4f-h in the way that they have, and how they can draw their conclusions from the data obtained. In the method, the authors state; 'The traction forces were analysed by summation of the force of a set of pillars perpendicular to the migrating cell axis'. They also have added a dotted yellow line to the right-hand image of Fig 4f (siRab18) - but not the left-hand image - to denote the migrating cell axis. But, if the dotted/solid line system that the authors have used is to be believed, the right-hand cell doesn't seem to necessarily be migrating in that direction. A more in-depth analysis of the influence of Rab18 and kinectin-1 on force distribution in migrating cells is needed to draw conclusions from these kinds of experiments.

6. I am struggling with the authors' description of the domain structure of kinectin-1. I thought that kinectin-1 comprised a short N-terminal domain (residues 1-6) within the ER lumen, a single transmembrane region (residues 7-29; denoted in green on the authors' diagram) spanning the ER membrane and a large C-terminal coiled coil cytoplasmic domain (residues 30-1357; depending on the splice variant) containing, amongst other things, the kinesin-binding site(s). So, I do not understand why the authors refer to the region 201-445 as one containing the transmembrane domain. Examination of the primary sequence of this region (201-445) does not indicate the presence of a region that could possibly conform to a canonical transmembrane domain. If I am correct, then the Rab18 binding site resides within the cytoplasmic domain of kinectin-1, where one would expect it to be, and not in the transmembrane domain. Is it possible for the authors to sort out this apparent misunderstanding?

7. Related to my previous point (6), the authors need to test whether purified Rab18 and kinectin (201-445) are able to interact directly. This would strengthen the mechanistic insight offered by the paper considerably.

8. I cannot find any information in the paper as to why the Rab18-GFP rescue construct seems to be resistant to the siRNA(s) used. Would it be possible for the authors to provide this? Also, this rescue experiments need to be performed more consistently throughout the study and not with just the selection that the authors have performed so far.

9. Fig3b - Since the cell size is significantly reduced between control and siRab18, it is expected that smaller cells contain fewer FAs. Therefore, it is necessary to normalise the number of FAs not only per cell, but per cell area. The results may show that FAs can still be formed but defects mainly happen during the maturing process.

10. Fig4d - The blot provided for this experiment is not convincing. For Rab18 siRNA the levels of pFAK decrease in the same manner as its loading control.
Reviewer #1 (Comments to the Authors (Required)):

The authors present some interesting but preliminary findings on a potential role for Rab18 and kinectin in cell migration. This extends previous studies by other workers showing that the ER contributes to cell migration, and that, in part, this is mediated by the ER integral membrane protein kinectin. In this study the authors provide some evidence that Rab18 binds to kinectin. However, how this alters kinectin function is unclear. Kinectin is already associated with the ER, so it is unlikely to be membrane recruitment. Focal adhesion dynamics are altered, but a direct mechanistic link from Rab18 and kinectin is not established. Therefore, the effects could be indirect and relate to alter ER morphology and dynamics, functions already associated with Rab18 and kinectin.

Following the suggestions of the reviewers, we now show that there is a direct mechanistic link between Rab18 and kinectin as the two proteins interact directly. We agree that Rab18 is not recruiting kinectin to the ER membrane, indeed our results indicate that the binding of Rab18 to kinectin is required to activate the kinesin-kinectin complex responsible for the anterograde ER movement to contact FA and promote their maturation.

The introduction section is poorly structured and does not make it particularly clear what ideas the authors are testing or what led them to investigate Rab18 and the role of the ER in cell migration. This is made worse since part of the introduction is in the first part of the results section. A major omission is the lack of introduction of published work on the role of the ER and kinectin in cell migration. In a number of instances, statements are gathered together and referenced as if all the citations support the points being made: "Indeed, Rab18 is also involved in the regulation of ER trafficking and structure as well as secretory granule transport (Dejgaard et al., 2008; Gerondopoulos 136 et al., 2014; Vazquez-Martinez et al., 2007).". These three publications make different claims. Dejgaard relates to ER-Golgi trafficking, Gerondopoulos relates to ER structure but not trafficking, and Vazquez-Martinez relates to secretory granules. This should be discussed and the information relevant for this study presented to the reader, not simply bundled together in this way.

We have now re-written the introduction following the reviewer's comments. We have also removed the first lines of the result section and mentioned in the introduction the published work on the role of the ER and kinectin in cell migration. Statements that were gathered together have been separated and now each of them clearly correspond to the proper reference(s).

Modest changes in cell migration behavior are seen in Rab18 knockdown cells after 21h. This seems reflect a general slowing down in wound closure. Comparison with other Rabs isn't performed, and this effect appears modest to some other previous studies where much larger effects were observed with endocytic recycling Rabs such as Rab4 and Rab11. The main text states that the rate of wound closure was reduced by over 20%. However, the graph in Figure 1b shows a delay in closure, but after that the slope of the curves looks similar. Looking at the data, we can conclude that the rate of closure is similar, but the extent of closure at 21h is reduced by ~15%. Since the migration of individual cells has not been tracked to measure velocity, I am not sure that "rate" should be used here. Figure 1d and 1e are important, but supplemental data.

We agree that the changes in cell migration behaviour in Rab18 knockdown cells are not as dramatic as those previously observed for endocytic recycling Rabs. However, it is not surprising that Rab18, which is an ER-associated Rab, has a minor effect on cell migration compared to endocytic recycling Rabs such as Rab4 and Rab11. In line with this, the delay in wound closure observed in cells stably
transfected with the KNT1 mutants unable to bind Rab18 (Suppl. Fig. 2a) is similar to the delay caused by Rab18 depletion.

In agreement with the reviewer’s comment, we have replaced “rate of closure” with “extent of wound closure”. The extent of wound closure in cells knockdown for Rab18 knocked down cells is reduced by 20% at 21 hours. Indeed, the % of relative wound density measured at 21h is 69.97% for the control and 55.49% (corresponding to 21% of the control) and 54.97% (corresponding to 22% of the control) for each of the two siRNAs against Rab18, respectively.

We have also tracked individual cell speed (Fig. 1d), confirming that knock down of Rab18 decreases cell velocity. The previous Figure 1d and 1e are now presented as supplemental data (suppl. Fig. 1a,b) as suggested.

Figure 1a. What percentage of cells are transfected for the rescue with GFP-Rab18? Normally this is <50%, which would be expected to limit recovery. Can the authors show the GFP channel? Did the GFP-positive cells migrate more than those not rescued?

The percentage of cells that are transfected for the rescue with GFP-Rab18 is around 30% in the wound healing experiments, where cells are seeded at higher percentage of confluence. We have now included the GFP channel in fig. 1a as requested even though in the combined image it is difficult to see the cells with low transfection efficiency. Therefore, we attach below for the referee, a zoomed picture where it is possible to appreciate also cells expressing Rab18 at lower levels.

We agree with the referee that the efficiency of transfection <50% explains the partial recovery observed in the rescue experiments of Fig. 1a,b. In line with this, the quantification of the single cell speed revealed that the GFP-positive cells migrate indeed with a velocity similar to the control cells and faster than the silenced ones (Fig. 1d).

The authors use U2OS cells. Are the results obtained seen with other cell lines? MDA-MB-231 cells have been used in another study of the role of kinectin and ER in cell migration, so it would be valuable to look at Rab18 in those cells. This would also enable the authors to look in more detail are the behavior of cell protrusions, and whether knockdown of Rab18 results in the loss of preferential orientation towards a chemoattractant.

We thank the referee for the suggestion. We have now performed chemotaxis experiments using MDA-MB-231 cells. The results show that in cells knocked down for Rab18 the preferential orientation towards the chemoattractant is lost, and that, in contrast to control cells, several non-oriented protrusions are formed (Fig. 8).
Figure 4h. Traction force should be in absolute units, not normalised. This hides variation in the control values. This is also an issue with the way data is presented in some other figures (see Figure 4e, 5f, 7b).

Traction force in absolute units (nN) was shown in figure 4g (Suppl. Fig. 1f in the revised version of the manuscript). Therefore, we have now removed the previous fig. 4h with the normalized values and to show the variation we have included the error bars and statistical analysis in the graph of Suppl. Fig. 1f. All the data in the manuscript are now presented not normalized to the control, including Fig. 5f (Fig. 5b in the revised version) and Fig. 7b (Fig. 5d in the revised version). The only figure where the results are presented relative to the control is Fig. 4e (Fig. 7e in the revised version), as this is a quantification of western blot bands. This is commonly done to ensure comparability between experiments, as it compensates for technical/non-sample related variations in signal intensity between experiments due, for example, to different exposure times.

What role does the Rab18-kinectin interaction play in FA dynamics and cell migration? The referenced Ng & Zhang papers indicate that ER spreading and kinectin and linked to migration. To extend this, the authors need to produce stable cell lines expressing different kinectin mutants defective for Rab18 binding and test these in the various assays used in Figures 1-4 for Rab18.

As suggested, we have generated U2OS stable cell lines expressing the kinectin mutants defective for Rab18 binding (delta 201-445 and K1), in addition to stable cell lines expressing the full length kinectin. We have also tested these cells in the following experiments:

- Suppl. fig. 2a: Wound healing (Fig. 1 in the previous version)
- Suppl. fig. 2b-c: Cell spreading (-Fig. 2 in the previous version)
- Suppl. fig. 2d-g: FA number and size (Fig. 3 in the previous version)
- Fig. 6: FA turnover (Fig. 4 in the previous version)

In addition, we have used these stable cell lines to study kinectin distribution between ER sheets and tubules (Fig. 4c-e).

Altogether, these results show that the kinectin mutants defective for Rab18 binding, and in particular the KNT1 delta 201-445 which specifically lacks the Rab18-binding site, result in a phenotype similar to the one obtained upon Rab18 depletion. This, together with the results from the direct interaction experiment (Fig. 3f), the ER-FA contact site formation (Fig. 5), the FRAP and chemotaxis data (Figs. 7 and 8), further extends the conclusion from the cited Ng & Zhang works. In particular, our results indicate that the Rab18 binding to kinectin is required to promote the ER transport towards the cell periphery to contact FAs and induce their maturation and therefore to sustain cell migration.

As already mentioned, examination of chemotaxis may also prove valuable.

We performed chemotaxis experiments and the results show that Rab18 knock down affects the orientation towards the chemoattractant (Fig. 8).

Different ER markers should be compared to the kinectin staining in control and Rab18 depleted cells for Figure 5c. Sheet and tubule compartments such as CLIMP63 and reticulons should be examined. This also needs to be extended to the localization of mutant kinectin constructs within the ER (Figure 6).

We have now compared kinectin distribution in sheet and tubule compartments using CLIMP63 and reticulon-3 as markers in control and Rab18 depleted cells. We have also analysed the distribution of kinectin delta 201-445 between these compartments. The results show that kinectin redistributes...
towards ER sheets both in cells silenced for Rab18 and in cells expressing the kinectin delta 201-445 mutant (Fig. 4c-e). This result further supports the role of Rab18-binding to kinectin in promoting kinectin-mediated transport towards the cell periphery.

We could not analyse the localization on the ER of the other kinectin mutant defective for Rab18 binding (K1), as this mutant has a cytosolic localization, as shown below, because it also lacks the transmembrane domain responsible for the insertion into the ER membrane:

There is also some slightly circular logic. If Rab18 and kinectin knockdown cells show cell spreading defects, then ER spreading will also be altered. This makes it difficult to be sure what is causal - is a cell spreading defect altering ER spreading, or is an ER defect altering cell spreading?

Cell spreading is initiated by the binding of the cell to the extracellular matrix, which triggers the recruitment of different FA proteins, leading to FAK activation and the formation of lamellipodia (Swaminathan et al., 2016; Theodosiou et al., 2016). According to this and to our results, it is likely that the defective ER spreading, which results in defective recruitment of FA proteins and in the decrease of FAK phosphorylation (Fig. 7), is the cause of the altered cell spreading.

Corrections:
1. Figure 5f. Graph Y-axis "FAas" I think should be "FAs".
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We thank the referee for the constructive comments. We have now analysed the time-lapse of the wound healing by single cell tracking. The results show that the cells silenced for Rab18 migrate slower than control cells and that introduction of GFP-Rab18 in knock down cells rescues the velocity defect (Fig. 1d).

It is true that reduced adhesion does not always lead to reduced cell migration. However, it is also reported that the speed of cell migration has a biphasic relationship with adhesion strength: fast migration occurs at intermediate adhesion strength, while low and high adhesion strengths promote slow migration (Gupton and Waterman-Storer, 2006). In addition, low adhesion strength can also result in defective protrusions, membrane ruffling and inefficient migration (Borm et al., 2005; Burridge and Wennerberg, 2004; Ng et al., 2016; Petrie et al., 2009). Cell protrusion formation requires indeed adhesion to the substratum, a step necessary before forward cell edge movement. The production of multiple randomly oriented protrusions in the presence of the chemoattractant in cells silenced for Rab18 (fig.8) is therefore in agreement with a model where the defective FA assembly/disassembly and maturation affects the spreading and the maintenance of correctly oriented protrusions similarly to what has been previously observed for KNT1 (Ng et al., 2016).
Indeed, FA maturation is required for maintenance of oriented protrusions during chemotaxis (Ng et al., 2016). FAs stabilize protrusions by linking the actin cytoskeleton to the extracellular matrix, and act as traction point as actomyosin contraction generates traction forces on the substratum. However, there is a complex, and not yet fully characterized, bidirectional interaction between FAs and actin cytoskeleton that makes difficult to investigate this process. Indeed, signalling from FAs influence cytoskeletal organization and actin polymerization, but also the cytoskeletal structures in turn influence the formation and disassembly of the adhesions. Our results from the micropillar experiments indicate a defective generation of traction forces that is consistent with the defect in FAs and in a possibly defective connection to the actin cytoskeleton. However, the investigation of the contribution of the actin cytoskeleton to the generation of traction forces, even though extremely interesting, is quite intricate and should rather be the object of a follow-up study.

2. The adhesion/migration-related approaches that the authors choose to use throughout the paper seems a little inconsistent. For instance, why have they looked at FA assembly/disassembly and contractility in Fig 4., but not subsequently? Similarly, why do the authors measure the effect of depletion of kinectin-1 and/or expression of Rab18-binding deficient mutants of kinectin-1 on FA/ER contact sites, but not on FA turnover and/or cell migration?

We have now extended the analysis of FA turnover (Fig. 6), cell migration, spreading, number and size of FAs (Suppl. Fig. 2), also to cells stably expressing the Rab18-binding deficient mutants of kinectin-1 delta 201-445 and K1.

3. As the authors mention, previous work on the role of kinectin and the ER in cell adhesion and migration analysed the dynamics of the ER with respect to cell protrusion and concluded that kinectin-mediated recruitment of the ER into extending lamellipodia facilitates the subsequent recruitment of FA proteins, such as β3 integrins and then vinculin (Zhang et al., 2010). Interestingly, one of the authors of this paper, Gareth Griffiths, is a departmental colleague of the authors of the present manuscript. Surely, if the authors believe that Rab18 is required for this process to occur, then they should test it experimentally, and provide some mechanistic data as to why this is so.

Following the reviewer’s suggestion, we performed FRAP analysis on β3 integrin and vinculin in presence or absence of Rab18. The results show that the fluorescence recovery of β3 integrins and vinculin at FAs after bleaching is significantly reduced in Rab18 knockdown cells and that the re-expression of Rab18 in those cells rescues the recovery kinetics (Fig. 7a,b). Intriguingly, the kinetics of β3 integrins and vinculin fluorescence recovery in cells silenced for Rab18 are similar to the kinetics measured in control cells in absence of contacts between FAs and ER (Fig. 7c). This indicates that these contacts are required for the recruitment of FA proteins for FA maturation.

We further show that the formation of the ER-FA contact sites required for FA maturation is regulated by the direct binding of Rab18 to kinectin, which promotes the kinesin-mediated anterograde transport of kinectin to FA sites (Figs. 3f, 4c-e, 5c,d).

4. The data in Fig. 3 are not convincing to this reviewer. I am not able to see evidence for a 'clear correlation between the two proteins, with both Rab18 and vinculin intensities increasing during FA assembly or decreasing during FA disassembly,'. This experiment needs to be conducted in a way that is statistically meaningful.

More data are now added to the original observation. The new graphs containing the mean intensity profiles ± s.e.m. from four independent live-cell imaging experiments are shown in Suppl. Fig.5.
5. I do not understand why the authors have chosen to perform the pillar experiment in Fig 4f-h in the way that they have, and how they can draw their conclusions from the data obtained. In the method, the authors state; 'The traction forces were analysed by summation of the force of a set of pillars perpendicular to the migrating cell axis'. They also have added a dotted yellow line to the right-hand image of Fig 4f (siRab18) - but not the left-hand image - to denote the migrating cell axis. But, if the dotted/solid line system that the authors have used is to be believed, the right-hand cell doesn’t seem to necessarily be migrating in that direction. A more in-depth analysis of the influence of Rab18 and kinectin-1 on force distribution in migrating cells is needed to draw conclusions from these kinds of experiments.

We agree entirely with the reviewer and are grateful for the input and the questions raised. We have replaced the single time point of migration direction and the mere reference to it with the trajectory obtained from centroid analysis - now plotted for the images of Fig. 4f (Suppl. fig. 1e in the revised version). While there is significant uncertainty in the image segmentation of transmitted light cell boundaries, the centroids are robust to small changes in segmentation methods. We have also provided more details in the material and methods section about how the traction forces were calculated. We hope that the more in depth data addresses the points without adding too much difficulty to the text. We did not investigate the evolution of the force vector under Rab18 vs kinectin as the directional measurement unlike its amplitude is either fairly noise or intricate and exceeds the scope of this paper.

6. I am struggling with the authors' description of the domain structure of kinectin-1. I thought that kinectin-1 comprised a short N-terminal domain (residues 1-6) within the ER lumen, a single transmembrane region (residues 7-29; denoted in green on the authors' diagram) spanning the ER membrane and a large C-terminal coiled coil cytoplasmic domain (residues 30-1357; depending on the splice variant) containing, amongst other things, the kinesin-binding site(s). So, I do not understand why the authors refer to the region 201-445 as one containing the transmembrane domain. Examination of the primary sequence of this region (201-445) does not indicate the presence of a region that could possibly conform to a canonical transmembrane domain. If I am correct, then the Rab18 binding site resides within the cytoplasmic domain of kinectin-1, where one would expect it to be, and not in the transmembrane domain. Is it possible for the authors to sort out this apparent misunderstanding?

The referee’s description of the domain structure of kinectin-1 is correct and we apologize if we were unclear in the text. We have now re-written this part (lines 270-283, page 6).

7. Related to my previous point (6), the authors need to test whether purified Rab18 and kinectin (201-445) are able to interact directly. This would strengthen the mechanistic insight offered by the paper considerably.

We thank the referee for the suggestion that we agree strengthens considerably the mechanistic insight. We purified both Rab18 and the kinectin (201-445) and were able to detect a direct interaction between these two proteins (Fig. 3f).

8. I cannot find any information in the paper as to why the Rab18-GFP rescue construct seems to be resistant to the siRNA(s) used. Would it be possible for the authors to provide this? Also, this rescue experiments need to be performed more consistently throughout the study and not with just the selection that the authors have performed so far.

We have now included in the material and methods the information about the siRNA resistant version of the GFP-Rab18 construct that we used in rescue experiments. We have also included
rescue experiments for the experiments in Fig. 3a,b (Fig. 2d-g in the revised manuscript), Fig. 5c,d (Fig. 4a,b in the revised manuscript), Fig. 4e,f (Suppl. fig. 4a-c in the revised manuscript) as well as in all the new experiments performed (Fig. 4c-e; Fig. 7 and Fig. 8).

9. Fig3b - Since the cell size is significantly reduced between control and siRab18, it is expected that smaller cells contain fewer FAs. Therefore, it is necessary to normalise the number of FAs not only per cell, but per cell area. The results may show that FAs can still be formed but defects mainly happen during the maturing process.

We have included the number of FA normalized per cell area as suggested (Fig. 2g in the revised manuscript). The results show that FAs can still be formed, and together with the FRAP data (Fig. 7) indicate that defects indeed happen during FA maturing process.

10. Fig4d - The blot provided for this experiment is not convincing. For Rab18 siRNA the levels of pFAK decrease in the same manner as its loading control.

A more representative blot is now shown in Fig. 7d (Fig. 4d of the previous version of the manuscript). In addition, we have confirmed this result also by IF analysis of pFAK (Fig. 7f).

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February 20, 2020

Re: JCB manuscript #201809020RR

Prof. Cinzia Progida
University of Oslo
Blindernveien 31
Oslo 0371
Norway

Dear Dr. Progida,

thank you for submitting your revised manuscript entitled "Rab18 regulates focal adhesion dynamics by interacting with kinectin-1 at the endoplasmic reticulum". The manuscript was re-reviewed by the original two reviewers, whose comments are appended to this letter. The manuscript does seem to be greatly improved and the re-review comments are supportive overall. However, the reviewers do raise some important points that we would like you to address in a final revision.

I have summarized the key remaining points below and made some suggestions of how to address these.

1) Both reviewer#1 and I share the same concern regarding the scratch wound data in figure 1 and in particular the strength of the new rescue data. The main concern with these data are that the Rab18 silencing effect is rather small and the rescue data are difficult to comprehend given that rescue in only a small number of cells somehow results in a full rescue with no obvious migration difference between rescued and silenced cells within the cell cohort (see also comment from reviewer#1). I would recommend the authors consider removing these data altogether and instead make a stronger emphasis on the new data on directional migration in chemotaxis where Rab18 silencing effects are bigger and the rescue complete.

2) It would be important to discuss the possible reasons for why in the micropilar experiments the authors observe reduced traction force at the beginning of the experiment, a traction force that seems to be recovered at the end of the time course in the Rab18 deficient cells.

3) Please consider removing the cell spreading data from the manuscript as suggested by reviewer#2.

4) Please consider addressing the other points made by reviewer#2 by discussing the suggested points/making textual edits and providing better example blots, is available.

Please also:
- Provide a short eTOC blurb
- Provide figures as separate, editable files according to the instructions for authors on JCB's website, paying particular attention to the guidelines for preparing images at sufficient resolution for screening and production
- Add a paragraph after the Materials and Methods section briefly summarizing the online supplementary materials
Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please note that I will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Johanna Ivaska, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have made a series of constructive responses to the original review comments, and added some important new data. The new data on directional migration in chemotaxis shows a relatively large effect when Rab18 is depleted. Importantly, this is fully rescued by the GFP-Rab18 re-expression. What is missing from this analysis is a link back to Rab18 interaction with kinectin. What is puzzling is the data in Figure 1. As noted in the original reviews, the effects of Rab18 depletion are slight in the scratch wound assay used by the authors. Adding the GFP-Rab18 channel to the figure only makes matters more confusing. If the cells in Figure 1C were rescued, and thus migrated more rapidly than the depleted cells surrounding them, then they should be enriched in the centre of the wound. This point was raised in my original review. However, the impression from the image shown is that GFP-Rab18 positive cells are in anything excluded from the central region. This isn't consistent with the hypothesis that migration is defective. Only a small percentage of cells are actually rescued in Figure 1A, so one would expect very little "rescue" in the assay. The graphed data (Figure 1B and image (Figure 1C right side) show near complete rescue.

Reviewer #2 (Comments to the Authors (Required)):

The authors have addressed many of my points and this has yielded a much stronger manuscript. In general, I feel that the paper is now acceptable for publication in the JCB. However, there are still some outstanding points. Some of these are ones that are incompletely resolved by the authors' revision, and others are points which have been thrown up by the new data. I feel that the authors
should address some of these, either by further discussion or (if possible) by limited further experimentation, and this should be at the editor's discretion.

Points completely dealt with:
The authors have dealt effectively and completely with my points Nos. 4, 5, 6, 7, 8 & 9

Points which need further discussion and/or limited experimentation:
1. The demonstration that cell migration speed is reduced following Rab18 depletion is convincing, and the experiments testing further the relationship between cell spreading, FA formation and cell migration seems to be gainful. However, the results from the micropillar experiments still need further discussion. In this, the authors observe reduced traction force at the beginning of the experiment, a traction force that seems to be recovered at the end of the time course in the Rab18 deficient cells. If maturation, rather than formation, of FAs is the suggested hypothesis for Rab18 role on cell migration, it seems strange that at later time points FA adhesions can promote a similar traction force to control cells. Especially, when the number of FAs per cell is significantly lower or not significantly changed per cell area. Is it possible for the authors to discuss this point?
2. The authors have now looked at a broader range of adhesion-related metrics (number and turnover of FAs, cell migration, and cell spreading) using the Rab18-binding deficient mutant of kinectin. However, I think that I would remove the cell spreading data from the paper as these are not convincing (the differences are so small and are probably not biologically relevant). Furthermore, the lack of effect of the KNT1 K1 mutant on cell spreading, despite its inability to bind to Rab18, doesn't help the authors' case.
3. The authors interestingly show the requirement of Rab18 expression for the recruitment of b3-integrin and vinculin to FAs that are in contact with the ER. Moreover, they show how subsequently pFAK levels are decreased in absence of Rab18. However, it is still unclear how integrin b3 is trafficking to the ER-FA contact sites to form FAs under the control of Rab18. The possibilities for how Rab18 influences this could be discussed. For instance, it is possible that Rab18 is controlling trafficking of integrin B3 back to the plasma membrane/FAs, and/or that the microtubules associated with the ER via KNT1 and Rab18 are required to traffic integrins to FAs.
10. The authors should provide representative images to accompany the pFAK quantification data in 7f.

Other points:
Fig. 3b (Fig. 5b in the first submission). Can the authors provide another example of a coloP experiment in which the loading of GFP-Rab18 and its mutants is more even? As it stands, Fig 3b is difficult to interpret because of the underloading of the dom. neg. and overloading of the CA. mutants.
Fig. 4a. The siRab18+GFP-Rab18 image looks to be in a more ventral plane that the other two conditions in this figure. If this is the case, then one would expect to see that the distribution of KNT1 was less concentrated in the perinuclear region. Can the authors please provide an image which is confirmed to be in the same Z plane of the control siRNA and siRab18 images?
Furthermore, the authors claim that redistribution of KNT1 to the perinuclear region after silencing Rab18 is due to a disruption of the KNT1 plus-end-mediated transport of the ER (lines 295-303). However, previous literature shows that Rab18 silencing indeed induces a reduction of the tubular structures of the ER, compensated by an increase on the sheet structures of the ER (Gerondopoulos 2014). The authors claim that the decrease of KNT1 on tubular structures after Rab18 silencing is owing to defects of KNT1 transport, but it might well be that the decrease of tubular structures by Rab18 itself is the cause for the decrease of the localisation of KNT1 in this ER compartment. This could be discussed.
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We agree with the reviewer and have mentioned in the discussion (lines 465-467) that silencing of KNT1 has been shown to also affect the maintenance of protrusions oriented toward the chemoattractant (Ng et al., 2016). This, in line with our results shown in figure 8, supports the proposed model where Rab18 and KNT1 regulate together the maintenance of oriented protrusions during chemotaxis.

What is puzzling is the data in Figure 1. As noted in the original reviews, the effects of Rab18 depletion are slight in the scratch wound assay used by the authors. Adding the GFP-Rab18 channel to the figure only makes matters more confusing. If the cells in Figure 1C were rescued, and thus migrated more rapidly than the depleted cells surrounding them, then they should be enriched in the centre of the wound. This point was raised in my original review. However, the impression from the image shown is that GFP-Rab18 positive cells are in anything excluded from the central region. This isn't consistent with the hypothesis that migration is defective. Only a small percentage of cells are actually rescued in Figure 1A, so one would expect very little "rescue" in the assay. The graphed data (Figure 1B and image (Figure 1C right side) show near complete rescue.

As mentioned in the previous revision, the cells expressing low/medium levels of GFP-Rab18 are difficult to visualize in the images shown in the figure 1a of the previous version due to the low sensitivity and resolution of the instrument used. However, as also suggested by the editor, we have now removed the data from the scratch wound assay.

Reviewer #2 (Comments to the Authors (Required)):

The authors have addressed many of my points and this has yielded a much stronger manuscript. In general, I feel that the paper is now acceptable for publication in the JCB. However, there are still some outstanding points. Some of these are ones that are incompletely resolved by the authors' revision, and others are points which have been thrown up by the new data. I feel that the authors should address some of these, either by further discussion or (if possible) by limited further experimentation, and this should be at the editor's discretion.

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Especially, when the number of FAs per cell is significantly lower or not significantly changed per cell area. Is it possible for the authors to discuss this point?

The traction force applied by the cells to move forward can be generated by small, nascent adhesions present at the leading edge rather than by mature FAs (Beningo et al., 2001). Indeed, force increases with size of adhesions only for larger adhesions, whereas no such correlation exists for smaller adhesions (>1μm²) (Tan et al., 2003). In line with this, the ability of Rab18 deficient cells to generate traction force, but with a delay compared to control cells, might not be linked to FA maturation but rather to a delay in nascent adhesion formation. Adhesion of a protruding cell edge is initiated by nascent adhesions, that assemble simultaneously to lamellipodial protrusion (Gardel et al., 2010). As Rab18 depletion affects protrusion orientation and delays cell spreading, it is likely that it also delays the formation of nascent adhesions. We have now summarized this in the discussion (lines 481-488).

2. The authors have now looked at a broader range of adhesion-related metrics (number and turnover of FAs, cell migration, and cell spreading) using the Rab18-binding deficient mutant of kinectin. However, I think that I would remove the cell spreading data from the paper as these are not convincing (the differences are so small and are probably not biologically relevant). Furthermore, the lack of effect of the KNT1 K1 mutant on cell spreading, despite its inability to bind to Rab18, doesn't help the authors' case.

We removed the cell spreading data for the Rab18-binding deficient mutants of kinectin, as suggested.

3. The authors interestingly show the requirement of Rab18 expression for the recruitment of β3-integrin and vinculin to FAs that are in contact with the ER. Moreover, they show how subsequently pFAK levels are decreased in absence of Rab18. However, it is still unclear how integrin β3 is trafficking to the ER-FA contact sites to form FAs under the control of Rab18. The possibilities for how Rab18 influences this could be discussed. For instance, it is possible that Rab18 is controlling trafficking of integrin B3 back to the plasma membrane/FAs, and/or that the microtubules associated with the ER via KNT1 and Rab18 are required to traffic integrins to FAs.

Following the reviewer's suggestion, we have now included in the discussion (lines 440-450) how Rab18 might control trafficking of β3-integrin to the plasma membrane/FAs. As it has been previously demonstrated that α5β3-integrin is also localized to the ER (Woods et al., 2004), Rab18 in complex with KNT1 and KIF5B could regulate the trafficking of the ER-localized α5β3-integrins to FAs along the microtubules. Alternatively, Rab18 might indirectly regulate the β3-integrin recycling by affecting the formation of ER-endosome contact sites. Indeed, the formation of ER-endosome contact sites influences endosomal fission, a processes dependent by the ER morphology (Rowland et al., 2014). As Rab18 has a critical role in the regulation of the ER morphology (Gerondopoulos et al., 2014), it might be involved in the process of ER-dependent endosomal fission and, in turn, in β3-integrin recycling to the plasma membrane.

10. The authors should provide representative images to accompany the pFAK quantification data in 7f.

As suggested, we added representative images related to the pFAK quantification (Figure 7d).

Other points:
Fig. 3b (Fig. 5b in the first submission). Can the authors provide another example of a coIP experiment in which the loading of GFP-Rab18 and its mutants is more even? As it stands, Fig 3b is difficult to interpret because of the underloading of the dom. neg. and overloading of the CA. mutants.

The loading of the GFP-Rab18 mutants in Fig. 3b (now Fig. 1b) is not perfectly even due to the transfection efficiency of the GFP-Rab18 dominant negative mutant. Indeed, a lower expression for this mutant compared to the wt was very often obtained. The blot presented in Figure 1b (Fig. 3b in the previous version of the manuscript) is the one among all the other experiments where the expression of the dominant negative mutant is actually more comparable to the wt.

Fig. 4a. The siRab18+GFP-Rab18 image looks to be in a more ventral plane that the other two conditions in this figure. If this is the case, then one would expect to see that the distribution of KNT1 was less concentrated in the perinuclear region. Can the authors please provide an image which is confirmed to be in the same Z plane of the control siRNA and siRab18 images?

As reported in the figure legend, all the images in Fig. 4a (now Fig. 2a) represent maximum intensity projections from Z-stacks and not a single confocal plane.

Furthermore, the authors claim that redistribution of KNT1 to the perinuclear region after silencing Rab18 is due to a disruption of the KNT1 plus-end-mediated transport of the ER (lines 295-303). However, previous literature shows that Rab18 silencing indeed induces a reduction of the tubular structures of the ER, compensated by an increase on the sheet structures of the ER (Gerondopoulos 2014). The authors claim that the decrease of KNT1 on tubular structures after Rab18 silencing is owing to defects of KNT1 transport, but it might well be that the decrease of tubular structures by Rab18 itself is the cause for the decrease of the localisation of KNT1 in this ER compartment. This could be discussed.

We agree with the reviewer that we cannot exclude that the decrease of tubular structures by Rab18 itself could be the cause for the decreased localization of KNT1 in this ER compartment. We have now included this in the discussion (lines 452-461), as suggested. However, also KNT1, similarly to Rab18, is involved in the regulation of the sheets:tubules ratio and ER extension to the cell periphery (Santama et al., 2004; Shibata et al., 2010). Furthermore, the expression of the KNT1 mutant defective for the Rab18-binding (KNT1 A201-445) causes defects both in the transport of KNT1 to ER tubular structures (Fig. 2 c-e) as well as in the ER transport to the FAs (Fig. 3c-d) in presence of Rab18. Altogether, this suggests that the cause for the altered KNT1 localization and ER transport might not be the lack of Rab18 by itself, but rather the impaired binding of KNT1 to Rab18.

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