Journal of Cell Science

Rabgap1 promotes recycling of active β1 integrins to support effective cell migration

Anna V. Samarelli, Tilman Ziegler, Alexander Meves, Reinhard Fässler and Ralph T. Böttcher
DOI: 10.1242/jcs.243683

Editor: Arnoud Sonnenberg

Review timeline
Original submission: 7 January 2020
Editorial decision: 6 February 2020
First revision received: 24 July 2020
Editorial decision: 10 August 2020
Second revision received: 13 August 2020
Accepted: 13 August 2020

Original submission

First decision letter

MS ID#: JOCES/2020/243683

MS TITLE: Rabgap1 promotes recycling of active β1 integrins to support effective cell migration

AUTHORS: Anna V. Samarelli, Tilman Ziegler, Alexander Meves, and Ralph T. Böttcher
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers consider your observation of interest, but at the same time raise a number of substantial points that prevent me from accepting the paper at this stage. Importantly, reviewer #1 finds the evidence that Rabgap1 acts uniquely on controlling Rab11 not sufficiently compelling and requests for some additional experiments and analyses. Specifically, he/she would like to know whether re-expression of wild-type Rabgap1, but not of an integrin-binding deficient mutant of Rabgap1 (i.e. a mutant carrying a single point mutation in the PTB domain of Rabgap1), in Rabgap1-deficient cells restores active β1 integrin recycling. Furthermore, because Rabgap1 acts on different Rabs, including Rab4, the reviewer wonders why this Rab GTPase only controls the trafficking of active integrins via the Rab11-dependent long loop and not that of inactive integrins via the Rab4-dependent short loop. Reviewer#2 and #3 raise somewhat related questions and feel that it is premature to attribute all the subsequent phenotypes on Rab11 when the activity of the other Rabs have not been investigated; their activities may have changed due to the overexpression of eGFP-Rabgap1.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This work identifies in Rabgap1 a critical RAB 11 GAP necessary for the regulation of active Beta1 integrin recycling. The work starts with the finding of an interaction between integrin NPxY motif and Rabgap1. Then the functional impact of Rabgap1 on integrin trafficking, spreading and cell migration is examined. Rabgap1 is found to localize to endosome and its silencing inhibits active beta1 integrin recycling likely through regulation of RAB 11 activity. Relevantly, Rabgap1 silenced cells are defective in cell spreading and wound closure in fibroblasts. Rabgap1 loss also impairs the invasion of MDA-MB-231 cells.

Comments for the author

Overall the data are of good quality and support the role of Rabgap1 in controlling a slow RAB11 recycling route of active beta1. Less compelling is the evidence in support of whether Rabgap1 acts uniquely on controlling RAB 11 and additional control experiments appear necessary. Specifically,
1. The authors identify the binding surface of the interaction between Integrin Beta1, NPxY783A and Rabgap1 PTB domain. They are, therefore, in a unique position to provide an experimental demonstration that this interaction is essential to control actin beta1 recycling. This could be done, for example, by re-expression of hRabgap1 (resistant to the silencing oligo) with a single point mutation(s) in residues of the PTB domain critical for interacting with the NPxY motifs.
2. Figure 4- The authors used GFP-hRABgap1 to restore the levels of the protein in knockdown cells and to test the amount of RAB11-GTP levels. The quality of the blot shown to document RAB 11-GTP levels, assuming is the best one obtained, is suboptimal, and not in line with the quantification measured in the graph of Fig. 4a.
3. Additionally and more importantly (as specified in point 1), the availability of hRabgap1 should be exploited to restore the defect in active beta integrin recycling.
4. The experiment depicted in Figure 4c-d that aims at showing that Rabgap1 acts through RAB 11 is interesting and potentially informative. However, the interpretation of the results obtained using RAB11-DN less straightforward than presented. Indeed, inhibition of RAB11 activity is expected to impair active beta1 recycling also in control cells regardless of whether Rabgap1 is expressed or not. Is this the case? In other words, an additional control necessary, here, is the measurement of the impact of RAB11-DN on active beta1 recycling kinetics.
5. Rabgap1 might be acting on different RABs, including Rab4, Rab6, Rab11, and Rab36. Hence it is unclear how it appears to specifically if not exclusively act on RAb11. Is the effect on Rab11 unique? The authors do show that the fast recycling route (largely RAB4- dependent) of inactive beta1 is not altered by the silencing of Rabgap1, arguing that in the context of integrin recycling Rabgap1 might act specifically on RAB11.
Whether this effect on beat1 integrin recycling is also responsible for the altered cell spreading, migration and invasion are also not clear. Thus, the author should be careful in interpreting the migration and cell spreading data simply as a consequence of the altered recycling of active beta1 intergrin. For example, they show that the silencing of Rabgap1 leads to increases focal adhesion size and number, possibly through the deregulation of Beta3 distribution. However, we are left with very little information as to how this could happen and how impairing beta1 recycling might lead to the redistribution of beta3 integrin. Some explanation is needed. Is there a rerouting of Beta3 after the impairment of Beta1 recycling as previously proposed?
6. Finally, the authors employed MDA-MB-231 to test the impact of Rasgap1 silencing on invasion, assuming the recycling of Beta1 is affected also in these cells. This, however, should be shown experimentally!!!

Very minor
LINE 107-HERE RABGAP1 is written in capital letters while this is not the case throughout the rest of the manuscript.

Reviewer 2

Advance summary and potential significance to field

This study identified Rabgap1 as a necessary factor for β1 integrin recycling. Interestingly, only active β1 integrins are targeted. It's also surprising that Rabgap1 attenuates Rab11 activity to promote recycling.

Comments for the author

It's clear that Rabgap1 associates with β1 integrins. Does Rabgap1 also function in cargo sorting besides its Rabgap activity? Since only active β1 integrins can be recycled by Rabgap1, is the interaction conformation-dependent? A co-IP experiment using conformation-specific β1 integrin antibody may address it.
Total β1 integrin levels on cell surface or in endosome keep intact upon Rabgap1 depletion. Are inactive β1 integrins be more actively recycled, or the active to inactive ratio of β1 integrins changed?
The finding of Rabgap1 attenuating Rab11 activity to support recycling needs further support. Can Rabgap1 catalytic-dead mutant rescue Rabgap1 knocking-down? How about Rab11 Q70L?

Reviewer 3

Advance summary and potential significance to field

In this interesting study the authors use quantitative mass spectrometry of Integrin -beta1 (ITGB1) mutants (Y783A) versus wildtype to identify Rabgap1 as a novel direct interaction partner of activated ITGB1. Rabgap1 depletion is shown to result in overactivation of Rab11, altered adhesion size and reduced migration and invasion rates. The current manuscript is a very good description of the effects of Rabgap1 depletion on ITGB1 trafficking. The possibility of internalised active ITGB1 affecting RabGTPase activation status and with it the endosomal system is a real conceptual advance. Very little is known about Rabgap1 and the existing literature is, as the authors state correctly, often contradictory. I think the authors need to investigate the Rab effectors downstream of Rabgap1 in more detail to avoid simplifying their phenotypes and attributing it all to Rab11 in the connected endosomal system, to fully support the exiting conclusions.

Comments for the author

-From Figure 1 onwards the authors use overexpression of eGFP-Rabgap1 as sole assay to confirm localisation of the protein in the endosomal system. They show that depletion of RabGAP1 seems to change trafficking of receptors and the activation status of RabGTPases.
Do verify that overexpression of EGFP-Rabgap1 does not alter the distribution of endosomal markers or activation status of RabGTPases themselves the author need to show control experiments testing the activation status of Rab4, 6, 11 in cells overexpressing RabGAP1. The activation stautus can either be tested directly or through measuring the endosomal trafficking rates of Rab4, 6 and 11 dependent cargo. Alternatively, they can stain the endogenous protein, but I assume there is no working antibody available?
For example, Figure4a does seem to show a reduction of Rab11 activity upon eGFP-Rabgap1 overexpression on knockdown background.

-The authors state correctly that RabGAP1 has been associated multiple targets in the Rab family of proteins (Rab 4, 6, 11, 36). Several of these Rabs have been shown to affect retrograde transport of receptors (Rab 4, 6, 11). From Figure 4 onwards the authors state that the effects they see are solely Rab11 mediated and show an experiment where relative internal levels of active integrins on a Rabgap1 knockdown background are the same as in wildtype cells with concomitant dominant-negative Rab11 overexpression. 1. This experiment should have cells without Rabgap1 knockdown as additional control to be able to make a better judgement of the effects of Rab11 overexpression. 2. Active integrins have been shown to take several recycling routes including late endosomes

(Dozynkiewicz et al. 2012). The authors solely focus on Rab11 and do not explain why they do not investigate Rab6 and Rab4 activity. I think it would be dangerous to pin all the subsequent phenotypes the authors see on Rab11 when the Rab6 and Rab4 activity have not been investigated. Therefore, this section needs to additionally evaluate Rab6 and Rab4 activity in Rabgap1 knockdown cells or the experiments in Figures 5 and 6 need to be rescued with dominant-negative Rab11 as well.

-Fig.2 needs single channel pictures to enable comparison of eGFP-Rabgap1 distribution. For example, the distribution of EGFP-Rabgap1 in C and E does not look very alike.

First revision

Author response to reviewers' comments

We would like to take the opportunity to thank all three reviewers and the editor for their fair and constructive review of our work which enabled us to improve our study. We addressed the issues raised by the reviewers and included this additional data into the revised manuscript.

Reviewer 1 Advance Summary and Potential Significance to Field:

This work identifies in Rabgap1 a critical RAB 11 GAP necessary for the regulation of active Beta1 integrin recycling. The work starts with the finding of an interaction between integrin NPxY motif and Rabgap1. Then the functional impact of Rabgap1 on integrin trafficking, spreading and cell migration is examined. Rabgap1 is found to localize to endosome and its silencing inhibits active beta1 integrin recycling likely through regulation of RAB 11 activity. Relevantly, Rabgap1 silenced cells are defective in cell spreading and wound closure in fibroblasts. Rabgap1 loss also impairs the invasion of MDA-MB-231 cells.

Reviewer 1 Comments for the Author:

Overall the data are of good quality and support the role of Rabgap1 in controlling a slow RAB11 recycling route of active beta1. Less compelling is the evidence in support of whether Rabgap1 acts uniquely on controlling RAB 11 and additional control experiments appear necessary. Specifically,

1. The authors identify the binding surface of the interaction between Integrin Beta1, NPxY783A and Rabgap1 PTB domain. They are, therefore, in a unique position to provide an experimental demonstration that this interaction is essential to control actin beta1 recycling. This could be done, for example, by re-expression of hRabgap1 (resistant to the silencing oligo) with a single point mutation(s) in residues of the PTB domain critical for interacting with the NPxY motifs.

We thank the reviewer for this suggestion as such experiments would strengthen the role of Rabgap1 for active β1 integrin recycling. As no structural information about the Rabgap1 PTB domain are available we analyzed different amino acids in the Rabgap1 PTB domain predicted to be involved in NPXY motif recognition and coordination (Uhlik et al., 2005) for changes in β1 integrin tail binding. We also included an inhibiting mutation of the Rabgap1 GAP function (Rabgap1 R612A) (Pan et al., Nature 2006). Substitution of F243 but not F217 of human Rabgap1 with alanine strongly reduced its ability to bind β1 integrin tail peptides while abrogating GAP activity of Rabgap1 (R621A) did not affect β1 integrin binding. This is included in the revised manuscript as new Figure 1F.
To test if the Rabgap1 GAP activity or its ability to interact with β1 integrin is important to regulate recycling of active β1 integrins, we followed the reviewer's suggestion and expressed shRNA-resistant GFP-tagged wild-type (wt), integrin-binding-deficient (F243A) or GAP-defective (R612A) human Rabgap1 in Rabgap1-depleted fibroblasts and determined the recycling rates after surface biotinylation. While wild-type Rabgap1 increased the recycling of active β1 integrin,

expression of Rabgap1 F243A and Rabgap1 R621A did not rescue the recycling defect, indicating that integrin binding and GAP activity are essential for Rabgap1 to regulate active β1 integrin recycling. These data are included in the revised manuscript as new Figure 3H,I.

Importantly, we tested the mutant Rabgap1 variants for their ability to rescue the migration and spreading phenotype of Rabgap1-depleted fibroblasts. Expression of shRNA-resistant GFP-tagged wild-type Rabgap1 in Rabgap1-depleted cells rescued cell spreading and migration to control levels, while the integrin-binding-deficient (F243A) or GAP activity-deficient (R612A) Rabgap1 did not - again supporting the finding that integrin binding and GAP activity are essential for Rabgap1 to regulate active β1 integrin recycling. These data are included in the revised manuscript as new Figure 5E,F.

2. Figure 4- The authors used GFP-hRABgap1 to restore the levels of the protein in knockdown cells and to test the amount of RAB11-GTP levels. The quality of the blot shown to document RAB 11- GTP levels, assuming is the best one obtained, is suboptimal, and not in line with the quantification measured in the graph of Fig. 4a.

We apologize for the poor quality of the initial blot. We have repeated the Rab11 activation assay two more times, added the quantifications to the previous data and plotted the data as single points to show the spread between individual experiments. We also exchanged the blots in Figure 4A to blots from an experiment that is more in line with the quantification measures. The additional experiments confirm that re-expression of GFP-tagged human Rabgap1 in Rabgap1 knockdown cells rescues Rab11-GTP (active Rab11) levels to control levels.

3. Additionally and more importantly (as specified in point 1), the availability of hRabgap1 should be exploited to restore the defect in active beta integrin recycling.

This is an important point. As indicated in our response to point 1, we identified a single amino acid point mutation within the Rabgap1 PTB domain (F243A) that prevented β1 integrin binding. Subsequently, we followed the reviewer's suggestion and expressed shRNA-resistant GFP-tagged wild-type (wt), integrin-binding-deficient (F243A) or GAP-defective (R612A) human Rabgap1 in Rabgap1-depleted fibroblasts and determined a) the recycling rates of active β1 integrin as well as spreading (b) and migration (c) rates of the different cell lines. Wild-type Rabgap1 increased the recycling of active β1 integrin and rescued rescued cell spreading and migration in Rabgap1-depleted fibroblasts to control levels. In contrast, expression of Rabgap1 F243A and Rabgap1 R621A did not rescue the recycling defect nor the spreading and migration phenotype. Together these data indicate that integrin binding and GAP activity are essential for Rabgap1 to regulate active β1 integrin recycling and integrin-mediated functions. These data are included in the revised manuscript as new Figure 3H,I and Figure 5E,F.

4. The experiment depicted in Figure 4c-d that aims at showing that Rabgap1 acts through RAB 11 is interesting and potentially informative. However, the interpretation of the results obtained using RAB11-DN less straightforward than presented. Indeed, inhibition of RAB11 activity is expected to impair active beta1 recycling also in control cells regardless of whether Rabgap1 is expressed or not. Is this the case? In other words, an additional control necessary, here, is the measurement of the impact of RAB11-DN on active beta1 recycling kinetics.

The reviewer is correct that Rab11 knockout or highly overexpression or Rab11a DN should impair the trafficking of active β1 integrin in the presence or absence of Rabgap1. We speculate that we did not detect strong alterations of active β1 integrin recycling in control cells since these cells are not Rab11 knockout and did not highly overexpress Rab11a DN. As a result, wild-type Rab11 is present and functional. Still, expression of Rab11 DN increased the levels of active β1 integrin in endosomes compared to Rab11a wt expressing cells from 46% to 62% (see Figure 4D). This is in line with the publication by Sahgal et al. which detected reduced active β1 integrin levels in endosomes after expression of Rab11a wt (Sahgal et al., JCS 2019). In contrast, the strong accumulation of active β1 integrin in endosomes in Rabgap1-depleted cells was normalized after expression of Rab11a DN (62% in control cells, 67% in Rabgap1-depleted cells) (Figure 4C, D). Overall, these data suggest that a fine-tuned Rab11 activity is crucial for proper recycling of active β1 integrins to the plasma membrane.

5. Rabgap1 might be acting on different RABs, including Rab4, Rab6, Rab11, and Rab36. Hence it is unclear how it appears to specifically if not exclusively act on RAb11. Is the effect on Rab11 unique? The authors do show that the fast recycling route (largely RAB4- dependent) of inactive beta1 is not altered by the silencing of Rabgap1, arguing that in the context of integrin recycling Rabgap1 might act specifically on RAB11.

The reviewer raises an important point. Our new data clearly show that the GAP activity is essential for Rabgap1 to regulate active β1 integrin recycling and integrin-mediated functions (Figure 3H,I and Figure 5E,F) suggesting that Rabgap1 functions through the regulation of a Rab GTPase. We focused on Rab11 since we observed the highest co-localization with GFP-tagged Rabgap1 and Rab11 is known to regulate the endosomal trafficking of active β1 integrins (Arjonen et al., Traffic 2012). Rab36 has not been studied in the context of integrin transport, Rab6 is involved in the retrograde-transport of inactive β1 integrin to the Golgi apparatus (Shafaq-Zadah et al., Nat Cell Biol 2016) and Rab4 mediates trafficking of inactive β1 integrin through the actin-dependent short-loop recycling pathway (Roberts et al., Curr Biol 2001).
Following the reviewers' concern, we tested Rabgap1-depleted fibroblasts for changes in Rab4 and Rab36 activity. We did not further study Rab6 because although Rabgap1 was initially described as GAP for Rab6 in in vitro experiments (Cuif et al., Embo J 1999) subsequent studies did not detect any effect of Rabgap1 on Rab6 function in cells and in GAP assays (Fuchs et al., JCB 2007; Kanno et al., Traffic 2010). GFP-tagged Rab36 protein is associated with the Golgi apparatus (Chen et al., Mol Membr Biol. 2010) and present in a specific class of endosomal structures distinct from late endosomes, lysosomes, early or recycling endosomes (Nottingham et al., JCB 2012). Rab36 function has been primarily linked to retrograde protein transport to the Golgi apparatus and the spatial distribution of late endosomes and lysosomes (Chen et al., Mol Membr Biol. 2010; Matsui et al., JBC 2012) and might therefore not be directly linked to the recycling of active β1 integrin from endosomes. Still, we analyzed the spatial distribution of late endosomes (Rab7) and lysosomes (Lamp1) by immunostaining with antibodies against Rab7 and Lamp1 in control and Rabgap1-depleted fibroblasts but did not detect any differences between the cell lines (Figure 1A for reviewer #1), indicating that Rab36 activity is not severely affected in Rabgap1-depeleted cells. Rab4A overexpression in MCF10A cells produced EEA1-positive enlarged endosomes that displayed prolonged and amplified EGF-induced EGFR-p1068 activation (Tubbesing et al., Mol Cancer Res 2020). While we did not detect changes in EEA1 localization or EEA1-positive endosome size (Figure 1A for reviewer #1) we observed a slightly prolonged EGF-induced EGFR-p1068 activation in Rabgap1-depleted cells which was rescued by re-expression of wild-type GFP-tagged Rabgap1 (Figure 1B for reviewer #1). This could be an indication for an overactivated Rab4A in Rabgap1-depleted cells.
Finally, to strengthen the involvement of Rab11 in Rabgap1-mediated recycling of active β1 integrin we tested if Rab11 functions downstream of Rabgap1 to regulate integrin-mediated cell spreading. Expression of dominant-negative but not wild-type Rab11a decreased cell spreading in Rabgap1-depleted fibroblasts to control levels suggesting that hyper-activation of Rab11 contributes to the Rabgap1 phenotype. This data is included in the revised manuscript as new Figure 5G.
As we cannot rule out the involvement of other Rabgap1-regulated GTPases we changed the manuscript text accordingly:

"Together, our results establish Rabgap1 as a regulatory element in the intracellular trafficking machinery required for α5β1 integrin recycling through its ability to regulate Rab GTPases activity, in particular Rab11."
"While we cannot rule out that other Rabgap1-regulated GTPases function in Rabgap1-mediated recycling of active β1 integrins, the rescue of intracellular active β1 integrin and cell spreading by expressing a dominant-negative variant of Rab11 in Rabgap1 knockdown cells suggest that hyper-activation of Rab11 contributes to the observed recycling phenotype."

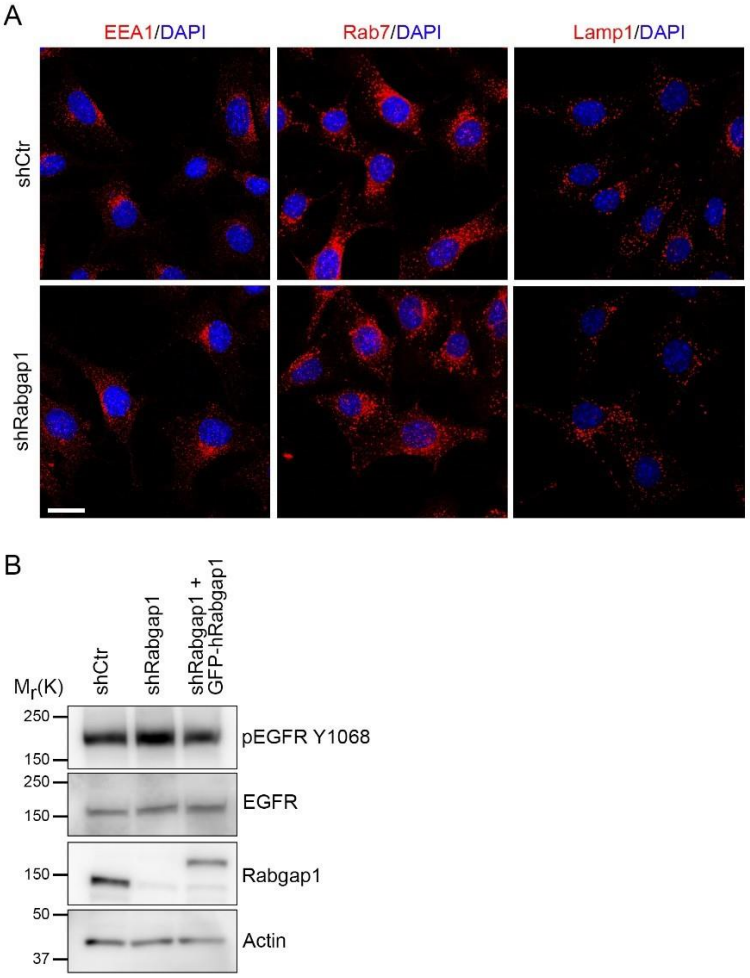

Figure 1 for reviewer #1: Effect of Rabgap1-depletion on endosomal marker localization and EGFR activation.
(A) Confocal images of control (shCtr) and Rabgap1-depleted (shRabgap1) fibroblasts stained with antibodies against EEA1, Rab7 or Lamp1. DAPI (blue) was used to stain nuclei. Scale bar, 20 µm. **(B)** Western blot analysis of control (shCtr), Rabgap1-depleted (shRabgap1) and Rabgap1-depleted fibroblasts after re-expression of wild-type GFP- tagged Rabgap1 after starvation and EGF treatment for 10 min at 37°C. Actin served as loading control.

Whether this effect on beat1 integrin recycling is also responsible for the altered cell spreading, migration and invasion are also not clear. Thus, the author should be careful in interpreting the migration and cell spreading data simply as a consequence of the altered recycling of active beta1 integrin. For example, they show that the silencing of Rabgap1 leads to increases focal adhesion size and number, possibly through the deregulation of Beta3 distribution. However, we are left with very little information as to how this could happen and how impairing beta1 recycling might lead to the redistribution of beta3 integrin. Some explanation is needed.
We agree with the reviewer that we cannot rule out that other processes, either Rabgap1-dependent or secondary effects, such as β3 integrin redistribution on the cell surface, might contribute to the cellular effects on cell migration and invasion. The new data -taking the reviewer's advice to analyze the function of integrin-binding-deficient (F243A) or GAP-defective (R612A) human Rabgap1 variants for active β1 integrin recycling as well as cell spreading and migration- clearly show connection between impaired recycling rates and migration defects (new Figure 3H,I and Figure 5E,F). We also show that expression of dominant-negative Rab11a rescues the intracellular accumulation of active β1 integrin and decreased cell spreading in Rabgap1-depleted fibroblasts to control levels (new Figure 5G). However, as other factors might contribute

to the cell migration and invasion phenotype, we included the following paragraph in the revised discussion: "The importance of Rabgap1 for cellular fitness is further emphasized by its ability to regulate cell spreading and to promote cell migration of mouse fibroblasts and cancer cell invasiveness. This requires a functional PTB domain and GAP activity and closely correlates with the ability of Rabgap1 to promote the recycling of active β1 integrins. Still, other Rabgap1-regulated processes and factors, such as β3 integrin redistribution on the cell surface, might contribute to the cellular effects on cell migration and invasion."

Is there a rerouting of Beta3 after the impairment of Beta1 recycling as previously proposed?
Although we measured a change in β3 integrin redistribution on the cell surface without altering in the total amount of β3 integrin on the cell surface of Rabgap1-depleted cells, we do not have any indication towards a re-routing of β3 integrin after impairment of active β1 integrin recycling. While β1 integrins have been shown to be re-routed after inhibiting β3 integrin (White et al., JCB 2007; Caswell et al., JCB 2008), β3 integrin internalization and recycling was not impaired in Ank2 KO cells exhibiting impaired polarized recycling of α5β1 integrins (Qu et al., Elife 2016). Unfortunately, our attempts to set up a recycling assay for β3 integrin using cell surface biotinylation and capture ELISA during these revisions were not successful. Due to too high background values the experiment-to-experiment variation were too high and we are not confident to include this data into the manuscript despite the fact that we did not detect significant differences in the recycling rates between control and Rabgap1-depleted fibroblasts.

6. Finally, the authors employed MDA-MB-231 to test the impact of Rasgap1 silencing on invasion, assuming the recycling of Beta1 is affected also in these cells. This, however, should be shown experimentally!!!
This is an important control experiment and we thank the reviewer for this suggestion. We used cell surface biotinylation and capture ELISA to determine the recycling rates of total α5β1 integrin and active (9EG7 positive) β1 integrins in MDA-MB-231 cells. Similar to fibroblasts, depletion of Rabgap1 by short hairpin RNA (shRabgap1) reduced the recycling of active but not total α5β1 integrin to the plasma membrane. This is included in the revised manuscript as new Figure 6A,B.

LINE 107-HERE RABGAP1 is written in capital letters while this is not the case throughout the rest of the manuscript.
This has been corrected in the revised manuscript.

Reviewer 2 Advance Summary and Potential Significance to Field:

This study identified Rabgap1 as a necessary factor for β1 integrin recycling. Interestingly, only active β1 integrins are targeted. It's also surprising that Rabgap1 attenuates Rab11 activity to promote recycling.

Reviewer 2 Comments for the Author:
It's clear that Rabgap1 associates with β1 integrins. Does Rabgap1 also function in cargo sorting besides its Rabgap activity? Since only active β1 integrins can be recycled by Rabgap1, is the interaction conformation-dependent? A co-IP experiment using conformation-specific β1 integrin antibody may address it.
The reviewer raises a very interesting question. Indeed, we write that "it is intriguing to speculate that Rabgap1 binding participates in the decision whether active integrins are recycled in a Rab11-dependent manner or routed into a degradative pathway." As suggested by reviewer #1 we screened and identified a single amino acid point mutation in the Rabgap1 PTB domain (Rabgap1 F243A) that strongly blocked the interaction of Rabgap1 with β1 integrin. This is included in the revised manuscript as new Figure 1F. Importantly, integrin-binding-deficient Rabgap1 F243A did not rescue the recycling defect nor the spreading and migration phenotype indicating that integrin binding is essential for Rabgap1 to regulate active β1 integrin recycling and integrin-mediated functions. These data are included in the revised manuscript as new Figure 3H,I and Figure 5E,F. Furthermore, we employed proximity-biotinylation assays using BioID (Kim et al., 2016) to verified the interaction of Rabgap1 with β1 integrin. Expression of the integrin α5-BioID2 fusion protein, mediating the biotinylation of proteins in proximity of the α5β1 integrin, allowed the pull-down of Rabgap1 in integrin β1 wt expressing fibroblasts but not in cells expressing the inactive β1 integrin

Y783A. This data is included in the revised manuscript as new Figure S1A. We also took the reviewers' advice and performed co-IP experiments using the conformation-specific 9EG7 antibody to IP active β1 integrins and reproducibly detected co-IPed Rabgap1 (Figure 2 for reviewer #2). [we have removed Figure 2, which was provided for the referees in confidence] *We decided against including this data into the revised manuscript as we did not have the tools for all required controls for our mouse fibroblast cells (e.g. conformation-specific antibodies recognizing the inactive β1 integrin conformation) to convincingly show the conformation-dependency of the β1 integrin/Rabgap1 interaction.*

Total β1 integrin levels on cell surface or in endosome keep intact upon Rabgap1 depletion. Are inactive β1 integrins be more actively recycled, or the active to inactive ratio of β1 integrins changed?

We believe that the main reason why we do not detect changes in the total β1 integrin levels on the cell surface and in endosomes is the fact that the majority of integrins in fibroblasts and cancer cells are in the inactive conformation whereas only a minor pool is in the active conformation. We do not have data for the fibroblasts used for this study but studies by the Ivaska and Bulleid labs have shown that in cancer cell lines (HT1080, NCI-H460, PC-3 and MDA-MB-231), only 10-20% of cell surface β1 integrins were in the active conformation (12G10- or 9EG7-positive) (Arjonen et al., Traffic 2012; Tiwari et al., JCS 2011). As a result, changes in this small active pool – although significant – do not lead to not readily detectable alterations when looking at the total pool of β1 integrins (which would be less then 5-10%). Unfortunately, conformation-specific antibodies recognizing the inactive β1 integrin conformation (such as mAb13, 1998, P1H5 and 4B4 mAbs) are not available for mouse β1 integrin and therefore we were not able to directly determine the internalization and recycling rates of inactive β1 integrins in our mouse fibroblasts cell system. Therefore, we can only speculate that the cell compensates for reduced active β1 integrin recycling by increased inactive active β1 integrin recycling or changing the ratio between active and inactive β1 integrin.

The finding of Rabgap1 attenuating Rab11 activity to support recycling needs further support. Can Rabgap1 catalytic-dead mutant rescue Rabgap1 knocking-down?

This is an important control experiment and we thank the reviewer for suggesting this experiment. We included an inhibiting mutation of the Rabgap1 GAP function by introducing a R621A mutant that abolish the IxxDxxR arginine finger motif (Pan et al., 2006). To test if the Rabgap1 GAP activity is important to mediate active β1 integrin recycling, we expressed shRNA-resistant GFP-tagged wild-type (wt) and GAP- defective (R612A) human Rabgap1 in Rabgap1-depleted fibroblasts and determined the recycling rates after surface biotinylation. While wild-type Rabgap1 rescued the recycling rates of active β1 integrin, expression Rabgap1 R621A did not rescue the recycling defect, indicating that GAP activity is essential for Rabgap1 to regulate active β1 integrin recycling. These data are included in the revised manuscript as new Figure 3H,I.
Importantly, we tested the mutant Rabgap1 variants for their ability to rescue the migration and spreading phenotype of Rabgap1-depleted fibroblasts. Expression of shRNA-resistant GFP-tagged wild-type Rabgap1 in Rabgap1-depleted cells rescued cell spreading and cell migration to control levels, while the GAP activity-deficient (R612A) Rabgap1 did not - again supporting the finding that GAP activity is essential for Rabgap1 to regulate active β1 integrin recycling. These data are included in the revised manuscript as new Figure 5E,F.

How about Rab11 Q70L?

We thank the reviewer for the question. Expression of wild-type Rab and a constitutively active form of this Rab frequently induce comparable phenotypic changes, e.g. Rab11 wt and the constitutively active Rab11 Q70L regulate the compartmentalization of early endosomes and the efficient transport from early endosomes to the trans-Golgi network (Wilcke et al., JCB 2000) or the expression of Rab22 wt or Rab2 Q64L for β1 and β3 integrin recycling (Qu et al., Elife 2016). We were including the Rab11a Q70L variant in some experiments but were struggling with low transfection rates of the Rab11a Q70L variant that prevented a detailed analysis.

Expression of Rab11a wt only marginally decreases (approx. 5%) the levels of active β1 integrin in

endosomes in control (shCtr) cells and had no effect in shRabgap1-depleted cells (compare Figure 3C and Figure 4D). The reduction in control cells is in line with the publication by Sahgal et al. which detected reduced active β1 integrin levels in endosomes after expression of Rab11a wt (Sahgal et al., JCS 2019). We assume that Rab11a wt overexpression in Rabgap1-depleted fibroblasts does not lead to changes in intracellular active β1 integrin levels due to the already elevated active Rab11 levels in these cells. Overall, these data suggest that a fine-tuned Rab11 activity is crucial for proper recycling of active β1 integrins to the plasma membrane.

Reviewer 3 Advance Summary and Potential Significance to Field:

In this interesting study the authors use quantitative mass spectrometry of Integrin-beta1 (ITGB1) mutants (Y783A) versus wildtype to identify Rabgap1 as a novel direct interaction partner of activated ITGB1. Rabgap1 depletion is shown to result in overactivation of Rab11, altered adhesion size and reduced migration and invasion rates. The current manuscript is a very good description of the effects of Rabgap1 depletion on ITGB1 trafficking. The possibility of internalised active ITGB1 affecting RabGTPase activation status and with it the endosomal system is a real conceptual advance. Very little is known about Rabgap1 and the existing literature is, as the authors state correctly, often contradictory. I think the authors need to investigate the Rab effectors downstream of Rabgap1 in more detail to avoid simplifying their phenotypes and attributing it all to Rab11 in the connected endosomal system, to fully support the exiting conclusions.

Reviewer 3 Comments for the Author:

-From Figure 1 onwards the authors use overexpression of eGFP-Rabgap1 as sole assay to confirm localisation of the protein in the endosomal system. They show that depletion of RabGAP1 seems to change trafficking of receptors and the activation status of RabGTPases.
Do verify that overexpression of EGFP-Rabgap1 does not alter the distribution of endosomal markers or activation status of RabGTPases themselves the author need to show control experiments testing the activation status of Rab4, 6, 11 in cells overexpressing RabGAP1. The activation status can either be tested directly or through measuring the endosomal trafficking rates of Rab4, 6 and 11 dependent cargo. Alternatively, they can stain the endogenous protein, but I assume there is no working antibody available?
For example, Figure4a does seem to show a reduction of Rab11 activity upon eGFP-Rabgap1 overexpression on knockdown background.

We thank the reviewer for this remark and suggestions. Unfortunately, we were not able to obtain an anti-Rabgap1 antibody for immunostainings despite testing different antibodies (Abcam ab153992, ABIN565136) with distinct fixation (PFA, MeOH) and permeabilization (Triton vs saponin) conditions and using Rabgap1-depleted cells as control. Possible GFP-Rabgap1 overexpression artefacts were of concern and we tried our best to rule them out. First, we generated stable GFP-Rabgap1-expressing cell lines and made sure to express the GFP-tagged Rabgap1 at endogenous protein levels as indicated in Figures 1F, 3H and 4A,B. With this expression levels we observed a rescue of Rab11 activity to control levels but did not observe a further reduction of Rab11 activity (Figure 4A). Due to the concern of reviewer #1 we have repeated the Rab11 activation assay twice more, added the quantifications to the previous data and plotted the data as single points to show the spread between individual experiments. We observed reduced Rab11-GTP levels in GFP-Rabgap1 expressing cells only once while in four other independent experiments Rab11-GTP levels were at or slightly above control levels (Figure 4A). In addition, we tested these cell lines for EGF receptor activation (as indication for Rab4 activation – further explanation see below) and also only detected a rescue to control levels (Figure 3A for reviewer #3). Second, we generated cell lines expressing GAP-activity-deficient GFP-tagged Rabgap1 (Rabgap1 R612A) and compared EEA1 and Rab7 localization with wild-type GFP-tagged Rabgap1 expressing cells (Figure 1C for reviewer). Modulation of Rab36 activity alters Rab7 and Lamp1 subcellular localization (Chen et al., Mol Membr Biol. 2010) and Rab4A overexpression in MCF10A cells produced EEA1-positive enlarged endosomes (Tubbesing et al., Mol Cancer Res 2020). However, we did not detect changes in the subcellular localization of these two endosomal compartments. This observation together with the Rab11 activity assay and EGF receptor activation suggestion that the GFP-hRabgap1 expression levels used in this study does not change Rab4, Rab11 and Rab36 activity.

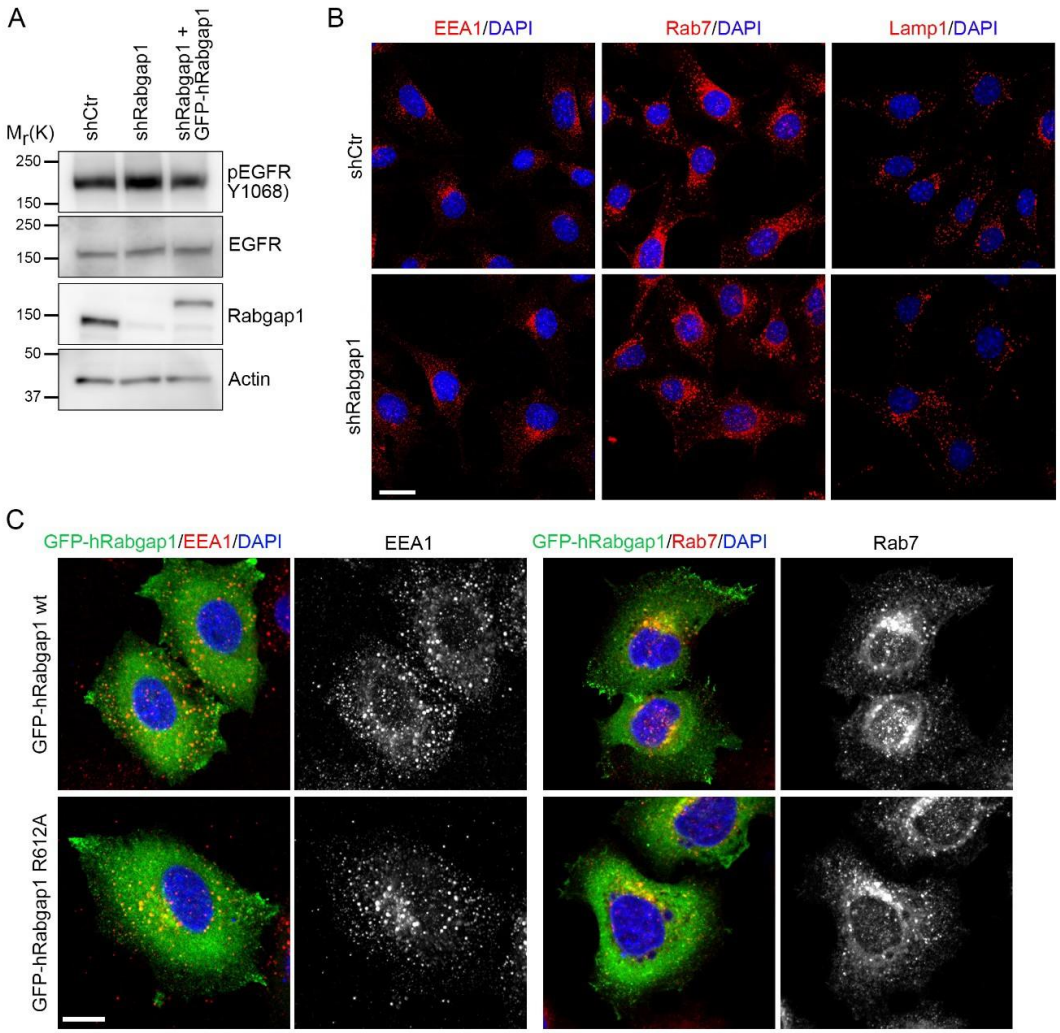

Figure 3 for reviewer #3: Effect of Rabgap1-depletion on endosomal marker localization and EGFR activation.
(A) Western blot analysis of control (shCtr), Rabgap1-depleted (shRabgap1) and Rabgap1-depleted fibroblasts after re-expression of wild-type GFP-tagged Rabgap1 after starvation and EGF treatment for 10 min at 37°C. **(B)** Confocal images of control (shCtr) and Rabgap1-depleted (shRabgap1) fibroblasts stained with antibodies against EEA1, Rab7 or Lamp1. DAPI (blue) was used to stain nuclei. Scale bar, 20 µm. **(C)** Confocal images of Rabgap1-depleted fibroblasts re-expressing GFP-tagged wild-type and GAP-defective (R612A) human Rabgap1 stained with antibodies against EEA1 or Rab7. DAPI (blue) was used to stain nuclei. DAPI (blue) was used to stain nuclei. Scale bar, 20 µm.

-The authors state correctly that RabGAP1 has been associated multiple targets in the Rab family of proteins (Rab 4, 6, 11, 36). Several of these Rabs have been shown to affect retrograde transport of receptors (Rab 4, 6, 11). From Figure 4 onwards the authors state that the effects they see are solely Rab11 mediated and show an experiment where relative internal levels of active integrins on a Rabgap1 knockdown background are the same as in wildtype cells with concomitant dominant-negative Rab11 overexpression. 1. This experiment should have cells without Rabgap1 knockdown as additional control to be able to make a better judgement of the effects of Rab11 overexpression.

We apologize to the reviewer that our writing might not have been precise. Indeed, we have expressed Rab11a DN in shRabgap1-depleted cells and in control cells. Expression of Rab11 DN increased the levels of active β1 integrin in endosomes compared to Rab11a wt expressing cells from 46% to 62% (see Figure 4D). This is in line with the publication by Sahgal et al. which detected reduced active β1 integrin levels in endosomes after expression of Rab11a wt (Sahgal et al., JCS

2019). In contrast, the strong accumulation of active β1 integrin in endosomes in Rabgap1-depleted cells was normalized after expression of Rab11a DN (reduction from 86% to 67% in Rabgap1-depleted cells; compared to 61% in control cells) (Figure 4C, D). Overall, these data suggest that a fine-tuned Rab11 activity is crucial for proper recycling of active β1 integrins to the plasma membrane.

2. Active integrins have been shown to take several recycling routes including late endosomes (Dozynkiewicz et al. 2012). The authors solely focus on Rab11 and do not explain why they do not investigate Rab6 and Rab4 activity. I think it would be dangerous to pin all the subsequent phenotypes the authors see on Rab11 when the Rab6 and Rab4 activity have not been investigated. Therefore, this section needs to additionally evaluate Rab6 and Rab4 activity in Rabgap1 knockdown cells or the experiments in Figures 5 and 6 need to be rescued with dominant-negative Rab11 as well.

We thank the reviewer for these comments and suggestions which were also raised by reviewer #1. In order strengthen the involvement of Rab11 in Rabgap1-mediated recycling of active β1 integrin we followed the reviewer's advice and tested if Rab11 functions downstream of Rabgap1 to regulate integrin-mediated cell spreading. Expression of dominant-negative but not wild-type Rab11a decreased cell spreading in Rabgap1-depleted fibroblasts to control levels suggesting that hyper-activation of Rab11 contributes to the Rabgap1 phenotype. This data is included in the revised manuscript as new Figure 5G.

We focused on Rab11 since we observed the highest co-localization with GFP-tagged Rabgap1 and Rab11 is known to regulate the endosomal trafficking of active β1 integrins (Arjonen et al., Traffic 2012). Rab36 has not been studied in the context of integrin transport, Rab6 is involved in the retrograde-transport of inactive β1 integrin to the Golgi apparatus (Shafaq-Zadah et al., Nat Cell Biol 2016) and Rab4 mediates trafficking of inactive β1 integrin through the actin-dependent short-loop recycling pathway (Roberts et al., Curr Biol 2001).
Following the reviewers' concerns, we tested Rabgap1-depleted fibroblasts for changes in Rab4 and Rab36 activity. We did not further study Rab6 because although Rabgap1 was initially described as GAP for Rab6 in in vitro assays (Cuif et al., Embo J 1999) subsequent studies did not detect any effect or Rabgap1 on Rab6 function in cells and in GAP assays (Fuchs et al., JCB 2007; Kanno et al., Traffic 2010). GFP-tagged Rab36 is associated with the Golgi apparatus (Chen et al., Mol Membr Biol. 2010). Rab36 function has been primarily linked to retrograde protein transport to the Golgi apparatus and the spatial distribution of late endosomes and lysosomes (Chen et al., Mol Membr Biol. 2010; Matsui et al., JBC 2012) and might therefore not be directly linked to the recycling of active β1 integrin from endosomes. We still analyzed the spatial distribution of late endosomes (Rab7) and lysosomes (Lamp1) by immunostaining with antibodies against Rab7 and Lamp1 in control and Rabgap1-depleted fibroblasts but did not detect any differences between the cell lines (Figure 3B for reviewer #3) indicating that Rab36 activity is not severely affected in Rabgap1-depeleted cells. Rab4A overexpression in MCF10A cells produced EEA1-positive enlarged endosomes that displayed prolonged and amplified EGF-induced EGFR-p1068 activation (Tubbesing et al., Mol Cancer Res 2020). While we did not detect changes in EEA1 localization or EEA1-positive endosome size (Figure 3B for reviewer #3) we observed a slightly prolonged EGF-induced EGFR-p1068 activation in Rabgap1-depleted cells which was rescued by re-expression of wild-type GFP-tagged Rabgap1 (Figure 3A for reviewer #3). This could be an indication for an overactivated Rab4A in Rabgap1-depleted cells.
As we cannot rule out the involvement of other Rabgap1-regulated GTPases we changed the manuscript text accordingly:
"Together, our results establish Rabgap1 as a regulatory element in the intracellular trafficking machinery required for a5β1 integrin recycling through its ability to regulate Rab GTPases activity, in particular Rab11."
"While we cannot rule out that other Rabgap1-regulated GTPases function in Rabgap1-mediated recycling of active β1 integrins, the rescue of intracellular active β1 integrin and cell spreading by expressing a dominant-negative variant of Rab11 in Rabgap1 knockdown cells suggest that hyper-activation of Rab11 contributes to the observed recycling phenotype."

-Fig.2 needs single channel pictures to enable comparison of eGFP-Rabgap1 distribution. For example, the distribution of EGFP-Rabgap1 in C and E does not look very alike.

Thank you for this suggestion. We changed Figure 2 in the revised manuscript accordingly. One reason for the different morphology of the cells between panels C and E is the fact that we included an acid wash step to remove antibodies remaining on the cell surface to analyze the co-localization between Rabgap1 and β1 integrin. This acid wash typically alters the cell morphology.

Second decision letter

MS ID#: JOCES/2020/243683

MS TITLE: Rabgap1 promotes recycling of active β1 integrins to support effective cell migration

AUTHORS: Anna V Samarelli, Tilman Ziegler, Alexander Meves, Reinhard Fässler, and Ralph T Böttcher
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, except for some minor issues, the reviewers find that you have satisfactorily addressed their comments and recommend publication. I guess you want to address these minor issues before submitting your final manuscript. Therefore, I am returning the manuscript for revision.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The revised work supports more cogently the role of Rabgap1 as a critical RAB 11 GAP necessary for the regulation of active Beta1 integrin recycling.

Comments for the author

The set of revised experiments performed do support the set of initial finding. Particularly relevant and convincing is the use of reconstitution experiments with RABgap1 mutant in either the PTB or the GAP domain.

Reviewer 2

Advance summary and potential significance to field

It is an interesting finding. The authors identified Rabgap1 as a critical Rab11 GAP to regulate the recycling of active beta1 integrin. Both GAP activity and direct interaction are essential for the function. The conclusion is convincingly supported by the evidence. I recommend its publication in Journal of Cell Science.

Comments for the author

The authors have properly addressed all my concerns.

Reviewer 3

Advance summary and potential significance to field

See previous review

Comments for the author

The authors have addressed all my queries and I have no further questions. The additional experiments performed by the authors to indirectly evaluate Rab4 and Rab7 activity in the RabGAP1 depleted cells are interesting and will improve the manuscript if included. The potential over-activation of Rab4 in RabGAP1 depleted cells, tested via EGFR p1068 levels, needs to be pointed out in text (apologies if already included and I missed it). My recommendation would be to include figure 3 for the reviewers as supplemental information in the manuscript, it will help readers in interpreting the data. Observations would only need to be quantified.

Second revision

Author response to reviewers' comments

We would like to take the opportunity to thank all three reviewers for their time and work. We addressed the issue raised by reviewer #3 and included this additional data with quantification into the revised manuscript.

Reviewer 1 Advance Summary and Potential Significance to Field:
The revised work supports more cogently the role of Rabgap1 as a critical RAB 11 GAP necessary for the regulation of active Beta1 integrin recycling.
Reviewer 1 Comments for the Author:
The set of revised experiments performed do support the set of initial finding. Particularly relevant and convincing is the use of reconstitution experiments with RABgap1 mutant in either the PTB or the GAP domain.
We thank the reviewer for the positive comment.

Reviewer 2 Advance Summary and Potential Significance to Field:
It is an interesting finding. The authors identified Rabgap1 as a critical Rab11 GAP to regulate the recycling of active beta1 integrin. Both GAP activity and direct interaction are essential for the function. The conclusion is convincingly supported by the evidence. I recommend its publication in Journal of Cell Science.

Reviewer 2 Comments for the Author:
The authors have properly addressed all my concerns.
We thank the reviewer for the positive comment.

Reviewer 3 Advance Summary and Potential Significance to Field: see previous review

Reviewer 3 Comments for the Author:
The authors have addressed all my queries and I have no further questions. The additional experiments performed by the authors to indirectly evaluate Rab4 and Rab7 activity in the RabGAP1 depleted cells are interesting and will improve the manuscript if included. The potential over- activation of Rab4 in RabGAP1 depleted cells, tested via EGFR p1068 levels, needs to be pointed out in text (apologies if already included and I missed it). My recommendation would be to include figure 3 for the reviewers as supplemental information in the manuscript, it will help readers in interpreting the data. Observations would only need to be quantified.

We thank the reviewer for this suggestion and included the Figure for the reviewers as new Supplemental Figure 3 into the revised manuscript. As recommended, we quantified the EGF receptor phosphorylation at Tyr1068 over three independent experiments.

Third decision letter

MS ID#: JOCES/2020/243683

MS TITLE: Rabgap1 promotes recycling of active β1 integrins to support effective cell migration

AUTHORS: Anna V Samarelli, Tilman Ziegler, Alexander Meves, Reinhard Fässler, and Ralph T Böttcher
ARTICLE TYPE: Research Article

Congratulations with a very nice paper.

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.