JCB

# An Arf/Rab cascade controls the growth and invasiveness of glioblastoma

Gopinath Kulasekaran, Mathilde Chaineau, Valerio Piscopo, Federica Verginelli, Maryam Fotouhi, Martine Girard, Yeman Tang, Rola Dali, Rita Lo, Stefano Stifani, and Peter McPherson

*Corresponding Author(s): Peter McPherson, McGill University*

## Review Timeline:

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## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

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May 26, 2020

Re: JCB manuscript #202004229

Dr. Peter S McPherson
McGill University
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Canada

Dear Dr. McPherson,

Thank you for submitting your manuscript entitled "An Arf/Rab cascade controls the growth and invasiveness of glioblastoma". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers all found the results interesting, but their support for the current data and scope varied. Two of the referees felt that the work provides a limited understanding of the cellular and molecular mechanisms by which Rab35 function influences tumor growth, cell migration/invasion, and tumor stem cell self-renewal. One referee felt that more mechanistic insight into how the Arf5/Rab35-regulation of SPOCD1 promotes cell proliferation and tumor growth was needed for publication. We have discussed the reviewers' remarks in-depth and would welcome the opportunity to consider a revision if you can tackle the reviews as follows.

- The points of Reviewers #1 and #3 should be attended to. They are minor, mostly addressable with language, but valid and relevant.

- The comments of Rev#2, our most critical referee, are pertinent and insightful. The technical points are valid (see also below) and should be addressed. We weigh in on the other points, requiring expansion of the findings and higher mechanistic definition of the processes, below:
Point 1. Please address this to the best of your ability. There is no need to measure proliferation and death. Using any single cell tracking methodology to prove increased motility would seem reasonable to us.
Point 2. Please address, for some additional in vitro experiments (no need to perform rescues for in vivo experiments). Please provide a control blot relative to Fig. 5H.
Point 3. We find these technical points relevant and valid and feel that all should be addressed.
Point 4. We agree with the referee that experiments establishing interactions in cells should be attempted and suggest you address this point, e.g., by co-ip.
Point 5. We agree with the referee, please address this point. At least one of the DENN domain should be used in all assays. We were also initially confused by the lack of consistency.
Point 6. This is a very relevant point in our view that is within the scope of your study. Please address this point experimentally.
Point 7. The referee makes an interesting point but in our view this is not a prerequisite for acceptance.
Point 8. The referee makes an interesting point but in our view this is not a prerequisite for

acceptance given the current scope of the study. Strengthening the core results around the Arf/Rab interactions and their functional implications with EGFR is in our view more central to the advance and of higher interest than expanding the study towards a better understanding of the connections to SPOCD1-related mechanisms.
Point 9. Please address with discussion.
Minor points should be addressed.

Please do not hesitate to contact us to discuss the revisions or if you have any questions, we'd be happy to discuss further as needed.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article 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: Articles may have up to 10 main text figures. 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.

***IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

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

Sincerely,

Pier Paolo Di Fiore, MD, PhD
Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

In this manuscript, Kulasekaran et al. report an original Arf5/Rab35 cascade in the context of tumor brain formation, using a combination of mouse models in vivo, cultured cells and in vitro assays. They found that Rab35 depletion in glioblastoma tumor initiating cells leads to an increase of brain tumor formation and a decrease of survival. Similar effects were observed upon depletion of Arf5, a protein that is found here to directly interact and to allosterically activate the endosomal Rab35 GEF DENND1/connecdenn. Furthermore, either Arf5 or Rab35 depletion promotes cell migration, invasion and selfrenewal in cultured cells. Finally, the pro-tumorigenic transcription factor SPOCD1 is found induced in Rab35-depleted tumors, likely as a result of sustained EGFR signaling in Rab35 or Arf5 depleted cells. The authors thus propose an Arf5□DENND1□Rab35 cascade that limits EGFR signaling, cell migration and invasiveness.

The experiments are very convincing, the paper is well written and the conclusions are solid. This work is original for two reasons: 1) it addresses for the first time the role of Rab35 in a mammalian, in vivo model and 2) it uncovers a new Arf/Rab cascade. I would therefore recommend publication in JCB. Addressing the following minor points would certainly improve the current manuscript.

Minor points

1. Could the authors provide quantification of the phenotype in Fig. 1B and 2A?

2. Fig. 1C/D: a zoom would be useful. Does Rab35 (or Arf5) depletion increase cell size, in addition to cell proliferation?

3. To reinforce the proposed Arf5/DENND1/Rab35 cascade, the authors should address whether the overexpression of DENND1 rescues the cellular defects observed upon Arf5 depletion (Fig. 5B, D and F). This could ideally be done in vivo but would require much more effort and is not essential.

4. Providing the list of up/down regulated genes in the RNAseq experiment would be useful (suppl. file).

5. The remarkable parallel between the Arf5/DENND1/Rab35 cascade (positive regulation, this paper) and the Arf6/TBC1D10B/Rab35 (negative regulation, PMID 26725203) that takes place at the levels of CCP/endosomes should be discussed in detail. In addition, the authors should speculate where the Arf5/DENND1 interaction occurs. Given the Arf5 localization, it is presumably when DENND1 is recruited, i.e. just after CCP scission from the plasma membrane.

A model depicting this two complementary cascades would be very useful in the final figure.

6. The authors should discuss what is already known for Rab35 in vivo, e.g. Drosophila (neuronal trafficking), C. elegans (trafficking) and Zebrafish (ciliogenesis). The present work is clearly a step ahead in mammalian biology and this could be emphasized.

7. When discussing the role of Rab35 in cadherin trafficking PMID 23197472 could be included.

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

The work of Kulasekaran et al. presents some interesting findings about Arf5 and its interaction with members of the DENND1/connecdenn family, which are GEFs for Rab35 (Marat and McPherson, 2010).
The authors show that Arf5 binding to the DENN domain of connecdenn enhances the GEF activity towards Rab35 and further suggest that this Arf5/Rab35 cascade might have a potential role in brain tumor growth.
This study extends previous works done by the same group and others where it was shown that loss of Rab35 enhances EGFR recycling and increases cell proliferation. They also previously demonstrated that the mRNA level of Rab35 is decreased in resected human glioblastoma (Allaire et al., 2013).
In the present study, Kulasekaran et al. provide some evidence that Rab35 knockdown is correlated with the upregulation of the tumor-promoting transcription factor SPOCD1, and suggest that this upregulation is a consequence of the EGFR activation caused by Arf5/Rab35 depletion. However, it is unclear how this is regulated. No evidence is provided to demonstrate how the EGFR activation may lead to SPOCD1 upregulation and whether this is a direct effect.
The identification of an Arf/Rab cascade that influences tumor growth is of interest to the readership of JCB. However, in terms of mechanistic insight the paper appears to be too preliminary for publication in this journal. The authors should provide more evidence and mechanistic insight for how Arf5/Rab35 regulates SPOCD1 to promote cell proliferation and tumor growth.
1) The cell migration data shown in Fig. 5 are not convincing. Multiple factors can contribute to different wound closure, including cell proliferation and cell death. As the authors also demonstrate with different experiments that Arf5/Rab35 depletion promotes cell proliferation and tumor growth, the increased cell migration seems to be rather a secondary effect caused by the enhanced proliferation. It should be better investigated if the accelerated wound healing is a consequence of the increased cell proliferation or primarily due to migration. Cell proliferation and cell death in the wound healing assay should be examined. Time-lapse microscopy followed by single cell tracking of cells migrating towards the wound would help to dissect the influence of Arf5/Rab35 on cell migration in more detail.
2) Rescue experiments are important and should be performed for the key phenotypes and not in only one experiment as the authors have performed so far. Furthermore, for the rescue experiment presented in Fig. 5H, a control blot showing GFP expression and representative images of the neurospheres should be provided.
3) The authors demonstrate by pull down experiments that the Rab35 GEF connecdenn/DENND1 binds Arf5. While in general they look nicely performed, some controls are missing. Full coomassie gels for the purified CD1 and CD2 DENN (including the mutants) proteins should be provided to show their purity.
Supp Fig.2A: Only the coomassie gel is shown for the Arf5 pulldown. The relative blot using an antibody against Arf5 should be presented as done for the other pull down experiments.

Supp Fig.2B: A blot showing the amount of GST-proteins in each sample should be included as control.
Fig. 3B-C: Control blots showing the transfection efficiency for the different ARFs are missing. This is important to exclude that the different binding observed for the different ARFs is not a consequence of a variability in the transfection efficiency.
4) The interactions are mainly proved by pull down of recombinant proteins. The authors should try to demonstrate the interaction also by immunoprecipitation of endogenous proteins, and Arf5 localization to Rab35-positive endosomes. This would strengthen the conclusion that Arf5 interacts with Rab35 GEFs in cells and that this Arf5/Rab35 cascade occurs in the endosomal pathway.
5) Pull down experiments with DENN domain mutants are only performed for CD2 DENN domain (Fig. 4B), while in vitro GEF assays are performed only for CD1 DENN domain (Fig. 4C-D). As CD1 and CD2 contains DENN domains with different levels of activity and ability to bind Rab35 (Marat and McPherson, 2010), the pull down experiments with DENN domain mutants and the in vitro GEF assay should be performed for both CD1 and CD2.
6) The blot in Fig. 8C indicates an increase in the total amount of EGFR upon Rab35 or Arf5 knockdown. It should be investigated whether Rab35 and Arf5 depletion promote EGFR activation by preventing or delaying EGFR degradation. Any defects resulting from Arf5 knockdown should then be rescued by Rab35 overexpression.
7) At the end of page 11, the authors speculate that the Arf5/Rab35 cascade is involved in the regulation of brain tumor growth through the enhanced recycling of EGFR. This hypothesis should be experimentally verified.
8) Furthermore, it should be investigated how the Arf5/Rab35 axis leads to the upregulation of SPOCD1, and some mechanistic data as to why this is so should be provided. Does Arf5 knockdown result in similar effects (upregulation of SPOCD1)? And if so, would Rab35 overexpression be able to rescue this?
9) The discussion is in the present version a summary of previously published results. The results presented in this paper should be included and discussed in relation to these previously published works.

Minor comments:
- Fig.1 D: The text indicating which samples are shown in the panels is missing
- Figure legend 2B (page. 33). BT205 instead of BT025

Reviewer #3 (Comments to the Authors (Required)):

This manuscript examines the role of Arf5 in the regulation of Rab35 in glioblastoma, focusing on the role of these proteins in brain tumor initiating cells (BTIC), using a variety of experimental models from in vivo cancer cell implantation, in vitro GTPase assays, protein interaction assays and cell-based assays. Implantation of the BTIC cell line BT025 into striatum revealed that silencing Rab35 increased tumor growth and deceased survival, while the reciprocal experiment of overexpressing Rab35 decreased tumor growth and prolonged survival. To better understand proteins that may be involved in regulation of Rab35, pull-down experiments using the DENN domain of connecdenn 1 and 2, known to function as GEFs for Rab35, were performed, revealing an interaction of this DENN domain with class II Arfs, in particular Arf5. Site-directed mutagenesis of the connecdenn 2 DENN domain indicated that mutations that impact substrate binding abolished interaction of this DENN domain with Rab35 but not Arf5. In addition, in vitro GTPgS binding assays revealed that the DENN domain had GEF activity towards Rab35 but not Arf5, indicating that DENN

likely does not function as a GEF for Arf5. As addition of Arf5 to the in vitro GEF assay with the DENN domain and Rab35 revealed enhanced GTPgS binding for Rab35, Arf5 may bind the DENN domain to enhance GEF activity towards Rab35.

Silencing of Rab35 and Arf5 similarly enhanced cell migration (increased scratch wound healing) and invasion (increased transit through Matrigel in transwell assays) in COS-7 cells. Silencing of Rab35 and Arf6 both similarly enhanced BTIC self-renewal, assessed by the growth of neurospheres. The increased BTIC self-renewal triggered by silencing of Arf5 was rescued by overexpression of Rab35, suggesting that Rab35 functions downstream of Arf5 to control BTIC self-renewal. Consistent with a role of Arf5 in a pathway regulating the GTP binding and activity of Rab35, silencing of Arf5 produced a similar phenotype as Rab35 in BTIC implantation assays in mouse brain (increased tumor growth). In addition, tumors derived from implantation of the more migratory BTIC cell line BT048 revealed that silencing of Arf5 or Rab35 increased tumor migration and invasion of cancer cells to the contralateral side of the brain.

To resolve how an Arf5-Rab35 signaling pathway may control tumor migration, invasion and growth, RNA-seq was performed on tumors dissected from mouse brains, comparing tumors derived from control BT205 BTIC cells to that derived from similar cells with Rab35 silencing. SPOCD1 was revealed to be the most highly upregulated gene in the Rab35 knockdown tumor cells. Follow-up experiments in U87 cells revealed that Rab35 silencing increased levels of SPODC1, as well as expression of PTX3 (previously shown to be controlled by SPODC1) and phosphorylation of the EGF receptor (previously shown to be regulated by Arf6, which also was shown to be regulated by Rab35 by this group).

Overall, this manuscript advances a novel model of the regulation of Rab35 by Arf5, involving a new molecular mechanism by which Arf5 binding to the DENN domain of the GEF connecdenn 2 enhances GEF activity towards Rab35. In this study, both Rab35 and Arf5 are revealed in this current study to have important novel functions in regulating brain tumor growth and invasion using in vivo animal models, and both are revealed to have important novel functions in regulation of cell migration and invasion, and in BTIC self-renewal using cell-based assays. In addition, the in vitro assays examining the regulation of Rab35 by Arf5 interaction with the DENN domain are well done and reveal important insight into the molecular mechanism. The manuscript is thus largely technically well executed, e.g. with care to use two different constructs for knockdown of Arf5 (it is not clear that knockdown-rescue experiments, as are often considered to show silencing specificity, are feasible here given complexity of some of the experiments, especially in vivo). These aspects of the manuscript are very strong, and the novel insight and conclusions are well supported by the data. This is an exciting discovery with a model that is well supported by the data.

Where the manuscript is somewhat weaker is in the understanding of the cellular or molecular mechanisms by which enhanced activity of Rab35 controls tumor growth, cell migration, cell invasion and tumor stem cell self-renewal. The RNA-seq approach reveals an important new candidate (SPOCD1) that is upregulated upon Rab35 silencing, but this is only one gene that exhibits altered expression in Rab35 silenced tumors. Providing a more comprehensive analysis of the gene expression alterations upon Rab35 silencing by including more of the analysis of the RNA-seq experiments would be very revealing for this manuscript. In addition, while the changes in SPODC1, PTX3 and EGFR downstream of silencing of Rab35 are intriguing, these changes are not linked functionally to cellular outcomes like migration, invasion and self-renewal that are examined in tis study. Including a functional analysis of SPODC1, PTX3 and EGFR, while interesting, is not critical for this manuscript to be complete. Similarly, while including more insight into the RNAseq data in this manuscript would be helpful, it is possible that a comprehensive analysis of the RNA-

seq data is better suited for a follow-up manuscript. If the latter, a more nuanced discussion of the possible mechanism by which Rab35 controls brain tumor cell outcomes is warranted, given that functional mechanisms for this are not yet clear. These weaknesses are not sufficient to dampen enthusiasm for this manuscript.

Some additional specific comments:

1) In the abstract, the phrase "... disruption of the Arf5/Rab35 axis in glioblastoma cells leads to strong activation of the epidermal growth factor receptor with resulting enhancement of SPODC1 levels", implies that SPODC1 levels are altered functionally downstream of enhanced EGFR signaling, which has not been demonstrated in this context.

2) The experiments shown in Figure 3 suggest a specific interaction of the DENN domain with class II Arf proteins, but not class I or III Arfs. This is based on the detection of GFP/CFP fusions of each Arf (expressed in HEK293 cells) in GST pull-down assays using HEK293 cell lysates. It would be helpful to include a western blot showing comparable levels of expression of the Arfs in HEK293 cell lysates. Also, C-terminal fusion of Arf proteins (as used here for expression of GFP/CFP-fused Arfs in HEK293 cells) may alter their activity or interactions, as was shown for Arf1 (PMID 20214751). If including an additional experiment with GST pull down of a subset of endogenous Arfs to support this specificity is not readily feasible, some tempering of the conclusion that class I and III Arfs do not interact with the DENN domain may be warranted. This does not detract from the conclusion that the DENN domain interacts with Arf5, which is the focus of this work and one of the major findings/conclusions.

3) In the results section, in reference to Figure 4C, "Remarkably, when we combined Arf5 with Rab35 we observed an enhancement in the GEF activity of the connecdenn DENN domain towards Rab35 (Fig. 4C)". The enhancement here is minor (~20%) - is this statistically significant (the experiment was performed 8 times according to the figure legend)? Notably, the subsequent panels (Fig 4E-F) show enhanced GEF activity of the DENN domain specifically towards Rab35 in the presence of Arf5 (with statistical analysis). As a result, this comment is not meant to challenge the overall conclusion, but how Fig. 4C itself can be directly interpreted.

4) The animal experiments in Figure 1-2 used different numbers of starting BTIC cells than in Figure 6. This is acknowledged in the text of the manuscript, but no reason is provided in the Results section (page 9). Could the authors provide a brief explanation of why the experiments in Figure 6 were performed with fewer BTIC cells at implantation?

5) The cell migration and invasion experiments (Figure 5A-C) were performed in COS-7 cells. It is not clear how cell migration and invasion of COS-7 cells relates to that of brain tumor cells. It is perhaps likely that technical limitations prevent similar migration experiments from being performed in brain cancer cells (for cell culture assays). However, that in vivo invasion of BT048 cells implanted in brain show enhanced invasion do indeed provide strong evidence that Rab35 and Arf5 control invasion in brain cancer cells, and largely mitigate this concern. It would perhaps be useful to better indicate that the experiments in Figure 5A-D were performed in COS-7 cells (ie in the Results text and not just the figure legend), especially since such care is taken to highlight cell type in most other experiments in the manuscript.

6) Arf5 may impact brain cancer cell outcomes independently of control of Rab35. Silencing of Arf5 reduces (~40-50%) but does not abolish Rab35 GTP binding (figure 4G-H). However, in many of the assays, knockdown of Arf5 produces an effect comparable to knockdown of Rab35, Knockdown of

Rab35 latter which appears to be very effective (e.g. Fig. 5A, 6A). The effect of silencing Arf5 on neurosphere growth is indeed rescued by Rab35 overexpression (Fig. 5E-H), but this effect may be specific to control of self-renewal. A very brief discussion of the possible contributions of Arf5 to brain tumor cell physiology independent of control of Rab35 may be warranted. This is a minor comment.

Institut-Hôpital neurologique de Montréal
Montreal Neurological Institute-Hospital

**Peter S McPherson**
PhD, FRSC

James McGill Professor
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McGill
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October 27, 2020

**Dr. Melina Casadio, Senior Scientific Editor**
**Dr. Pier Paolo Di Fiore, Editor**
**Journal of Cell Biology**

Dear Melina and Pier Paolo:

Thank you very much for your review of our manuscript (#202004229), “An Arf/Rab cascade controls the growth and invasiveness of glioblastoma". We especially wish to thank you for your detailed analysis of the reviewer’s comments and for providing guidance on addressing the comments. As described in detail below, we have now addressed all of the issues raised by the reviewers, in many cases through the addition of new data.

Sincerely

Peter McPherson, PhD, FRSC

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**Response to reviewer 1**

**Editor comments)** “The points of Reviewers #1 and #3 should be attended to. They are minor, mostly addressable with language, but valid and relevant”.

**Reviewer 1, comment 1)** “Could the authors provide quantification of the phenotype in Fig. 1B and 2A”?

**Response to reviewer 1, comment 1**) The experiments in Fig. 1B and 2A were designed primarily to test for the possibility of altered tumor growth and to examine for potential changes in survival. Thus, for quantification, we focused on the survival data presented in Figs. 1E and 2B/C, which are, in essence, quantification of tumor growth and are the relevant outcome of enhanced or decreased growth of the tumors following Rab35 knockdown or overexpression, respectively. In Figs. 6B-D and 7B-D we provide quantified analysis of the size of the brain tumors and the migration of brain tumor cells to the contralateral side of the brain following knockdown of both Arf5 and Rab35.

**Reviewer 1, comment 2)** “Fig. 1C/D: a zoom would be useful. Does Rab35 (or Arf5) depletion increase cell size, in addition to cell proliferation”?

**Response to reviewer 1, comment 2)** We have included a zoom for Fig. 1D. We do not see any obvious evidence for a change in cell size following Rab35 or Arf5 depletion. Moreover, we have examined cells in culture in various experiments in which we have depleted Rab35 or Arf5 and have not observed obvious changes in cell size, although this was not necessarily the focus of the experiments.

**Reviewer 1, comment 3)** “To reinforce the proposed Arf5/DENND1/Rab35 cascade, the authors should address whether the overexpression of DENND1 rescues the cellular defects observed upon Arf5 depletion (Fig. 5B, D and F). This could ideally be done in vivo but would require much more effort and is not essential”.

**Response to reviewer 1, comment 3)** We agree with the reviewer and made numerous attempts to clone DENND1 into lentiviral vectors for expression/rescue studies. Unfortunately, these were not successful. In the original submission we did describe experiments demonstrating that Rab35 expression rescues alterations in self-renewal resulting from Arf5 knockdown (Fig. 5I/J and Fig. S3E/F of the revised manuscript) and we have added new data demonstrating that Rab35 also rescues alterations in cell migration resulting from Arf5 knockdown (Fig. 5D/E and Fig. S3A of the revised manuscript), further confirming the Arf5/Rab35 cascade.

**Reviewer 1, comment 4)** “Providing the list of up/down regulated genes in the RNAseq experiment would be useful (suppl. file)”.

**Response to reviewer 1, comment 4)** This point was also raised by reviewer 3 in comment 1. We firmly believe that a comprehensive analysis of the RNAseq data would be better served in a follow up manuscript, a sentiment echoed by reviewer 3. As noted in response to reviewer 2, comments 6-8, and presented in Figs. 9A/B of the revised manuscript, we now provide new data demonstrating a more comprehensive functional link between Rab35 knockdown, increased levels of activated EGF receptor, and upregulation of SPOCD1 levels. Specifically, we have demonstrated that the upregulation of SPOCD1 levels caused by knockdown of Rab35 can be reversed by addition of Erlotinib, a specific

EGF receptor tyrosine kinase inhibitor, placing SPOCD1 upregulation firmly downstream of Rab35 knockdown-induced EGF receptor activation. This data further confirms the validity of the RNAseq data.

**Reviewer 1, comment 5)** "The remarkable parallel between the Arf5/DENND1/Rab35 cascade (positive regulation, this paper) and the Arf6/TBC1D10B/Rab35 (negative regulation, PMID 26725203) that takes place at the levels of CCP/endosomes should be discussed in detail. In addition, the authors should speculate where the Arf5/DENND1 interaction occurs. Given the Arf5 localization, it is presumably when DENND1 is recruited, i.e. just after CCP scission from the plasma membrane. A model depicting this two complementary cascades would be very useful in the final figure".

**Response to reviewer 1, comment 5)** We agree with the reviewer and believe that the Arf5/DENND1/Rab35 cascade is taking place on early CCP/endosomes. This alters recycling of the EGF receptor, leading to enhanced EGF receptor levels and activation, cascading to SPOCD1 upregulation. As described in response to comment 4 above, and comments 6-8 of reviewer 2, we have added new data (Figs. S5B-D and Figs. 9A/B of the revised manuscript) further supporting this model. We have added a graphical representation of this model as revised Fig. 9C. and furthered our discussion on these issues on page 12/13 of the revised manuscript. Finally, we have made reference to PMID 26725203.

**Reviewer 1, comment 6)** "The authors should discuss what is already known for Rab35 in vivo, e.g. Drosophila (neuronal trafficking), C. elegans (trafficking) and Zebrafish (ciliogenesis). The present work is clearly a step ahead in mammalian biology and this could be emphasized".

**Response to reviewer 1, comment 6)** We agree with the reviewer that the discussion was lacking some fundamental aspects of Rab35 biology. We have expanded the discussion along the lines proposed by the reviewer.

**Reviewer 1, comment 7) "**When discussing the role of Rab35 in cadherin trafficking PMID 23197472 could be included".

**Response to reviewer 1, comment 7)** We agree with the reviewer and have added PMID 23197472 in regards to our discussion of cadherin trafficking.

**Response to reviewer 2**

**Reviewer 2, comment 1)** "The cell migration data shown in Fig. 5 are not convincing. Multiple factors can contribute to different wound closure, including cell proliferation and cell death. As the authors also demonstrate with different experiments that Arf5/Rab35 depletion promotes cell proliferation and tumor growth, the increased cell migration seems to be rather a secondary effect caused by the enhanced proliferation. It should be better investigated if the accelerated wound healing is a consequence of the increased cell proliferation or primarily due to migration. Cell proliferation and cell death in the wound healing assay should be examined. Time-lapse microscopy followed by single cell tracking of cells migrating towards the wound would help to dissect the influence of Arf5/Rab35 on cell migration in more detail".

**Editor comment**) “Point 1. Please address this to the best of your ability. There is no need to measure proliferation and death. Using any single cell tracking methodology to prove increased motility would seem reasonable to us”.

**Response to reviewer 2, comment 1)** We thank the reviewer for the comment and the editor for the clarity. We have now performed time lapse imaging with single cell tracking and provide quantification for cell migration, in terms of distance travelled (revised Fig. S3B and) and velocity (revised Fig. S3C). We find that knockdown of both Rab35 and Arf5 significantly increase the migration of single cells. We note that the increased migration seen in the single cell tracking experiments and the wound healing assays are consistent with the enhanced invasion of cells both in cell culture experiments (revised Fig. 5F) and in the *in vivo* studies (revised Fig. 7B-D).

**Reviewer 2, comment 2)** “Rescue experiments are important and should be performed for the key phenotypes and not in only one experiment as the authors have performed so far. Furthermore, for the rescue experiment presented in Fig. 5H, a control blot showing GFP expression and representative images of the neurospheres should be provided”.

**Editor comment**) “Please address, for some additional in vitro experiments (no need to perform rescues for in vivo experiments). Please provide a control blot relative to Fig. 5H”.

**Response to reviewer 2, comment 2)** We agree with the reviewer and have added a new rescue experiment for the cell migration assays. We demonstrate that Rab35 expression rescues the enhanced migration resulting from Arf5 knockdown (revised Fig. 5D/E). For this experiment we include a control blot (revised Fig. S3A) and we have added a control blot (revised Fig. S3H) and representative images of the neurospheres (revised Fig. S3G) for original Fig. 5H (now Fig. 5J).

**Reviewer 2, comment 3)** “The authors demonstrate by pull down experiments that the Rab35 GEF connecdenn/DENND1 binds Arf5. While in general they look nicely performed, some controls are missing. Full coomassie gels for the purified CD1 and CD2 DENN (including the mutants) proteins should be provided to show their purity. Supp Fig.2A: Only the coomassie gel is shown for the Arf5 pulldown. The relative blot using an antibody against Arf5 should be presented as done for the other pull down experiments. Supp Fig.2B: A blot showing the amount of GST-proteins in each sample should be included as control. Fig. 3B-C: Control blots showing the transfection efficiency for the different ARFs are missing. This is important to exclude that the different binding observed for the different ARFs is not a consequence of a variability in the transfection efficiency”.

**Editor comment**) “We find these technical points relevant and valid and feel that all should be addressed”.

**Response to reviewer 2, comment 3)** We agree with the reviewer and editor. For Fig. 3 we have added full ponceau stained transfers corresponding to each blot (revised Fig. 3A-C/D). For Supp Fig. 2A, we have added a blot using antibody against Arf5 as suggested (revised Fig. S2B). For Supp Fig. 2B (now Fig. S2C) we have added a blot showing the amount of GST-protein in each sample (revised Fig. S2D). For Fig. 3B-C we have added control blots to demonstrate transfection efficiency (revised Fig. 3D).

**Reviewer 2, comment 4)** “The interactions are mainly proved by pull down of recombinant proteins. The authors should try to demonstrate the interaction also by immunoprecipitation of endogenous

proteins, and Arf5 localization to Rab35-positive endosomes. This would strengthen the conclusion that Arf5 interacts with Rab35 GEFs in cells and that this Arf5/Rab35 cascade occurs in the endosomal pathway".

**Editor comment**) "We agree with the referee that experiments establishing interactions in cells should be attempted and suggest you address this point, e.g., by co-ip".

**Response to reviewer 2, comment 4)** We attempted co-immunoprecipitation studies that ultimately were not successful. This may reflect somewhat low affinity (or related) high off-rates. We do however note that the physiological significance of the Arf5/Rab35 cascade rests on more than just the protein/protein interactions, which are further confirmed by mutational studies of the DENND1A/B DENN domains (Figs. 3A/B and Figs. S1D/E of the revised manuscript). Specifically, in revised Figs. 5-8 and related supplementary Figures, we demonstrate that Arf5 and Rab35 knockdown phenocopy in terms of influence on cell migration, cell invasion, self-renewal, tumor growth in brains, tumor cell migration in brains, and EGF receptor activation, supporting that the two proteins are on a related cell biological pathway. Dr. Cassanova has previously demonstrated that Arf5 localizes to clathrin-coated structures (Moravec *et al*., 2012) as does Rab35 (Allaire et al., 2010; Cauvin et al., 2016) and DENND1 (Allaire et al., 2006; Cauvin et al., 2016).

**Reviewer 2, comment 5)** "Pull down experiments with DENN domain mutants are only performed for CD2 DENN domain (Fig. 4B), while in vitro GEF assays are performed only for CD1 DENN domain (Fig. 4C-D). As CD1 and CD2 contains DENN domains with different levels of activity and ability to bind Rab35 (Marat and McPherson, 2010), the pull down experiments with DENN domain mutants and the in vitro GEF assay should be performed for both CD1 and CD2".

**Editor comment)** "We agree with the referee, please address this point. At least one of the DENN domain should be used in all assays. We were also initially confused by the lack of consistency".

**Response to reviewer 2, comment 5)** We agree with the reviewer and editor and have performed new experiments in which we introduce the point mutations based on the CD2 DENN domain structure into CD1. Both the triple and quadrupole mutations that disrupt Rab35 binding in CD2 have the same effect in CD1 (revised Figs. S1D/E). In neither case is there any influence on Arf5 binding (revised Figs. 4A/B and revised Figs. S1D/E). We also performed an experiment to assess the potential nucleotide dependency of Arf5 interaction with CD1. As we had already observed for Arf5 interaction with CD2 (revised Fig. S1F/G), there is no nucleotide selectivity for Arf5 interaction with CD1 (revised Figs. S1H/I).

**Reviewer 2, comment 6)** "The blot in Fig. 8C indicates an increase in the total amount of EGFR upon Rab35 or Arf5 knockdown. It should be investigated whether Rab35 and Arf5 depletion promote EGFR activation by preventing or delaying EGFR degradation. Any defects resulting from Arf5 knockdown should then be rescued by Rab35 overexpression"

**Editor comment)** "This is a very relevant point in our view that is within the scope of your study. Please address this point experimentally".

**Response to reviewer 2, comment 6)** We agree and have performed a new experiment to address this issue. Specifically, we treated control or Rab35 knockdown cells with cycloheximide and examined the steady-state levels of EGF receptor before and following 16h of drug treatment. The increase in

EGF receptor levels at steady-state resulting from Rab35 knockdown are partially reversed by cycloheximide treatment (revised Figs. S5B-D). However, the relative degree of decrease in EGF receptor levels appear even less in the Rab35 knockdown cells than the control cells, a fact borne out when the decreases in EGF receptor levels in control and Rab35 knockdown cells following drug treatment are normalized to the levels in non-treated cells (revised Fig. S5D). Given that blocking new EGF receptor synthesis only partially reverses the increase in EGF receptor levels, and that the decrease is even less than in the non-treated cells, we reasoned that the increase does not result from enhanced EGF receptor synthesis and thus results from decreased degradation, likely representing increased recycling.

**Reviewer 2, comment 7) "**At the end of page 11, the authors speculate that the Arf5/Rab35 cascade is involved in the regulation of brain tumor growth through the enhanced recycling of EGFR. This hypothesis should be experimentally verified".

**Editor comment)** "The referee makes an interesting point but in our view, this is not a prerequisite for acceptance".

**Reviewer 2, comment 8)** "Furthermore, it should be investigated how the Arf5/Rab35 axis leads to the upregulation of SPOCD1, and some mechanistic data as to why this is so should be provided. Does Arf5 knockdown result in similar effects (upregulation of SPOCD1)? And if so, would Rab35 overexpression be able to rescue this"?

**Editor comment)** "The referee makes an interesting point but in our view this is not a prerequisite for acceptance given the current scope of the study. Strengthening the core results around the Arf/Rab interactions and their functional implications with EGFR is in our view more central to the advance and of higher interest than expanding the study towards a better understanding of the connections to SPOCD1-related mechanisms".

**Response to reviewer 2, comment 7 and 8)** These are related comments so we respond to them together. We have performed a new experiment that advances the relationship between the Arf5/Rab35 axis, EGF receptor function, and SPOCD1 expression. Specifically, we knockdown Rab35 and find, as reported in Fig. 8 of the original manuscript, that there are enhanced levels and activation of the EGF receptor, and that the levels of SPOCD1 are increased. We then treat the cells with Erlotinib, a specific EGF receptor tyrosine kinase inhibitor. This reverses both EGF receptor activation and the Rab35-dependent increase in SPOCD1 levels. This provides evidence that SPOCD1 is upregulated downstream of EGF receptor activation, a result that has not been previously reported. The new data is found in Figs. 9A/B of the revised manuscript. Taken together, we now suggest a model in which Arf5 activates the GEF activity of DENND1 towards Rab35 early in the endocytic process (on clathrin-coated pits or vesicles or early endosomes). This leads to enhanced EGF receptor recycling and receptor activation. The receptor signals to an increase in SPOCD1 levels, driving enhanced self-renewal and tumor growth. Following from the suggestion of reviewer 1, comment 5, we have added a model (revised Fig. 9C) demonstrating this hypothesis.

**Reviewer 2, comment 9) "**The discussion is in the present version a summary of previously published results. The results presented in this paper should be included and discussed in relation to these previously published works.
Minor comments:
- Fig.1 D: The text indicating which samples are shown in the panels is missing

- Figure legend 2B (page. 33). BT205 instead of BT025”

**Editor comment)** “Please address with discussion. Minor points should be addressed”.

**Response to reviewer 2, comment 9)** The text has been added to the panels in the figure. BT205 has been corrected to BT025 (it’s sort of funny, BT205 is our lab room number).We apologize to the reviewers in that the discussion was not well fleshed out. We have modified the discussion according to both this comment and the comments of reviewer 1.

**Response to reviewer 3**

**Editor comments)** “The points of Reviewers #1 and #3 should be attended to. They are minor, mostly addressable with language, but valid and relevant”.

**Reviewer 3, comment 1)** “Where the manuscript is somewhat weaker is in the understanding of the cellular or molecular mechanisms by which enhanced activity of Rab35 controls tumor growth, cell migration, cell invasion and tumor stem cell self-renewal. The RNA-seq approach reveals an important new candidate (SPOCD1) that is upregulated upon Rab35 silencing, but this is only one gene that exhibits altered expression in Rab35 silenced tumors. Providing a more comprehensive analysis of the gene expression alterations upon Rab35 silencing by including more of the analysis of the RNA-seq experiments would be very revealing for this manuscript”.
“In addition, while the changes in SPODC1, PTX3 and EGFR downstream of silencing of Rab35 are intriguing, these changes are not linked functionally to cellular outcomes like migration, invasion and self-renewal that are examined in tis study. Including a functional analysis of SPODC1, PTX3 and EGFR, while interesting, is not critical for this manuscript to be complete”.
“Similarly, while including more insight into the RNAseq data in this manuscript would be helpful, it is possible that a comprehensive analysis of the RNA-seq data is better suited for a follow-up manuscript. If the latter, a more nuanced discussion of the possible mechanism by which Rab35 controls brain tumor cell outcomes is warranted, given that functional mechanisms for this are not yet clear. These weaknesses are not sufficient to dampen enthusiasm for this manuscript”.

**Response to reviewer 3, comment 1)** Several of the points raised in this comment were echoed by reviewer 1 and 2. As discussed in response to reviewer 1, comment 4, a comprehensive analysis of the RNA-seq data will be better suited for a follow up study. However, as discussed in detail in response to comments 7 and 8 of reviewer 2, and presented in Figs. 9A/B of the revised manuscript, we now provide new data regarding the link between Rab35 knockdown, increased levels of activated EGF receptor, and upregulation of SPOCD1 levels. Specifically, we demonstrate that the upregulation of SPOCD1 resulting from Rab35 knockdown is reversed by treatment of cells with a specific EGF receptor kinase inhibitor, Erlotinib. This further validates the identification of SPOCD1 as upregulated in the RNA-seq data, and suggests a model that enhanced EGF receptor recycling and activation, resulting from Rab35 activation in the early endosomal pathway, signals to increased levels of SPOCD1, driving, at least in part, tumor phenotypes. Following from the suggestion of reviewer 1, comment 5, we have added a graphical representation of this model (revised Fig.9C).

**Reviewer 3, comment 2)** “In the abstract, the phrase "... disruption of the Arf5/Rab35 axis in glioblastoma cells leads to strong activation of the epidermal growth factor receptor with resulting enhancement of SPODC1 levels", implies that SPODC1 levels are altered functionally downstream of enhanced EGFR signaling, which has not been demonstrated in this context”.

**Response to reviewer 3, comment 2)** We agree with the reviewer that in the original submission, this statement was too strong. However, given the new data described in response to comment 1 of this reviewer, and comments 7/8 of reviewer 2, we feel this statement is now appropriate.

**Reviewer 3, comment 3)** "The experiments shown in Figure 3 suggest a specific interaction of the DENN domain with class II Arf proteins, but not class I or III Arfs. This is based on the detection of GFP/CFP fusions of each Arf (expressed in HEK293 cells) in GST pull-down assays using HEK293 cell lysates. It would be helpful to include a western blot showing comparable levels of expression of the Arfs in HEK293 cell lysates".

**Response to reviewer 3, comment 3)** We agree and have included the requested western blot (revised Fig. 3D).

**Reviewer 3, comment 4)** "Also, C-terminal fusion of Arf proteins (as used here for expression of GFP/CFP-fused Arfs in HEK293 cells) may alter their activity or interactions, as was shown for Arf1 (PMID 20214751). If including an additional experiment with GST pull down of a subset of endogenous Arfs to support this specificity is not readily feasible, some tempering of the conclusion that class I and III Arfs do not interact with the DENN domain may be warranted. This does not detract from the conclusion that the DENN domain interacts with Arf5, which is the focus of this work and one of the major findings/conclusions"

**Response to reviewer 3, comment 4)** We present immunoblots demonstrating the affinity selection of purified Arf5, in which we have removed the tag, with DENN domains of both DENND1A and DENND1B (revised Fig. S1A/B). Moreover, we note however that there has been arguments made that C-terminal tagging of Arfs allows for greater functionality (Montagnac et al., 2011; Bottanelli et al., 2017). That said, we agree with the reviewer and have tempered our conclusions regarding the lack of binding of class I and III Arfs to the DENN domain (pg. 6 of the revised manuscript).

**Reviewer 3, comment 5)** "In the results section, in reference to Figure 4C, "Remarkably, when we combined Arf5 with Rab35 we observed an enhancement in the GEF activity of the connecdenn DENN domain towards Rab35 (Fig. 4C)". The enhancement here is minor (~20%) - is this statistically significant (the experiment was performed 8 times according to the figure legend)? Notably, the subsequent panels (Fig 4E-F) show enhanced GEF activity of the DENN domain specifically towards Rab35 in the presence of Arf5 (with statistical analysis). As a result, this comment is not meant to challenge the overall conclusion, but how Fig. 4C itself can be directly interpreted".

**Response to reviewer 3, comment 5)** A graph corresponding to Fig 4C is included in the revised manuscript (Fig. 4D). This data reveals that when we combine Arf5 with Rab35, there is significant enhancement in the GEF activity of the connecdenn DENN domain towards Rab35.

**Reviewer 3, comment 6)** "The animal experiments in Figure 1-2 used different numbers of starting BTIC cells than in Figure 6. This is acknowledged in the text of the manuscript, but no reason is provided in the Results section (page 9). Could the authors provide a brief explanation of why the experiments in Figure 6 were performed with fewer BTIC cells at implantation"?

**Response to reviewer 3, comment 6)** The experiments in Fig. 1 and 2 of the original manuscript were designed primarily to test for the possibility of altered tumor growth and to examine survival. Large numbers of cells were used to shorten the time spans needed to generate the survival curves. The experiments in Figs. 6 and 7 were designed to more quantitatively measure tumor growth and to examine for potential alterations in cell migration. Thus, smaller numbers of cells were used to allow for slower development of the tumor, providing more controlled growth with animal sacrificed and a pre-determined period. We have added this justification on page 10 of the revised manuscript.

**Reviewer 3, comment 7)** "The cell migration and invasion experiments (Figure 5A-C) were performed in COS-7 cells. It is not clear how cell migration and invasion of COS-7 cells relates to that of brain tumor cells. It is perhaps likely that technical limitations prevent similar migration experiments from being performed in brain cancer cells (for cell culture assays). However, that in vivo invasion of BT048 cells implanted in brain show enhanced invasion do indeed provide strong evidence that Rab35 and Arf5 control invasion in brain cancer cells, and largely mitigate this concern. It would perhaps be useful to better indicate that the experiments in Figure 5A-D were performed in COS-7 cells (ie in the Results text and not just the figure legend), especially since such care is taken to highlight cell type in most other experiments in the manuscript".

**Response to reviewer 3, comment 7)** We apologize for this oversight and have noted the use of COS-7 cells on page 9 of the results section.

**Reviewer 3, comment 8)** "Arf5 may impact brain cancer cell outcomes independently of control of Rab35. Silencing of Arf5 reduces (~40-50%) but does not abolish Rab35 GTP binding (figure 4G-H). However, in many of the assays, knockdown of Arf5 produces an effect comparable to knockdown of Rab35, Knockdown of Rab35 latter which appears to be very effective (e.g. Fig. 5A, 6A). The effect of silencing Arf5 on neurosphere growth is indeed rescued by Rab35 overexpression (Fig. 5E-H), but this effect may be specific to control of self-renewal. A very brief discussion of the possible contributions of Arf5 to brain tumor cell physiology independent of control of Rab35 may be warranted. This is a minor comment".

**Response to reviewer 3, comment 8)** Arf5 clearly has many functions outside of its activation of Rab35, and we have thus added a brief discussion (pg. 15 of the revised manuscript) regarding a potential role for Arf5 in brain tumor development independent of its influence on Rab35. We thank the reviewer for this suggestion.

**Reference**

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November 16, 2020

RE: JCB Manuscript #202004229R

Dr. Peter S McPherson
McGill University
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Montreal, Quebec H3A 2B4
Canada

Dear Dr. McPherson,

Thank you for submitting your revised manuscript entitled "An Arf/Rab cascade controls the growth and invasiveness of glioblastoma". The reviewers enthusiastically recommend publication. We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

1) Titles, eTOC: Please consider the following revision suggestions aimed at increasing the accessibility of the work for a broad audience and non-experts.

Running title (50 characters max, including spaces): an Arf/Rab cascade controls glioblastoma growth

eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.
- Please include a summary statement on the title page of the resubmission. It should start with "First author name(s) et al..." to match our preferred style.

2) Figure formatting:
Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.
- Please be sure to add unit labels to all panels.

3) S5D legend is mislabelled as C.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.
Please indicate n/sample size/how many experiments the data are representative of: 6D

5) Materials and methods: Should be comprehensive and not simply reference a previous

publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.
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- Please include species and source for all antibodies, including secondary, as well as catalog numbers/vendor identifiers if available.
- Sequences should be provided for all oligos: primers, si/shRNA, gRNAs, etc., even negative controls, if made available to you from the manufacturer, *even if described in other published work*
- Please provide more detail about the lentivirus-mediated knockdown of Rab35, viral particle production procedures, and the control non-targeting shRNAmiR virus - even if described in other published work.
- More information about single-cell migration analysis is needed, even if described in other published work.
- Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
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b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

6) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.
- Please include one brief sentence per item.

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Sincerely,

Pier Paolo Di Fiore, MD, PhD
Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

The authors have adequately addressed many of my previous comments and concerns.

Reviewer #3 (Comments to the Authors (Required)):

1. This work examines the role of the endocytic regulator Rab35 in brain cancer, focusing on cell migration and invasion. Rab35 negatively regulates brain tumor growth upon implantation of brain tumor initiating cells in mice. Mechanistic studies identified that the Rab35 GEF connecdenn proteins bind Arf5, and that Arf5 binding to DENN domains promotes GEF activity towards Rab35. Perturbations of Arf6 and Rab35 impacted cell migration in cultured cells, consistent with the effects of Rab35 perturbation in tumor growth in mouse brains. Rab35 silencing resulted in enhanced expression and activation of EGFR, which in turn controlled the induction of SPOCD1. This study thus links regulation of endocytic traffic by Rab35 and Arf6 to regulation of gene expression and control of EGFR, which in turn correlates with a role for Rab35 in promoting brain

cancer.

2. This revised manuscript is much improved, and all the points raised by this reviewer have been at least adequately addressed. The major point raised for the initial submission was that there was no examination of the functional consequence of EGFR upregulation upon Rab35 silencing. The revised manuscript now provides evidence that treatment with erlotinib, an EGFR inhibitor, blocks the increase in SPOCD1 upon Rab35 silencing. While a demonstration of the role of SPOCD1 in the effects that occur upon Rab35 silencing would be insightful, in my view this is beyond the scope of the current study. Including this new data in Figure 9 linking EGFR function to changes in gene expression of SPOCD1 upon Rab35 silencing is quite novel, in that it provides a novel mechanism by which changes in endocytic membrane traffic can impact gene expression.

The evidence that Rab35 is a potent regulator of tumor growth in mouse brains upon implantation of brain tumor initiating cells is strong, as this is supported by complementary effects of Rab35 silencing and overexpression conditions. This is well supported by the data, is very novel, and is one of the major strengths of this work.

The in vitro experiments showing that Arf5 binds to the DENN domain, but that this interaction does not result in the DENN domain having GEF activity for Arf5 is supported by binding assays involving DENN mutants, and by assays that probe GTP binding. This is well supported by the data, is very novel, and is another of the major strengths of this work.

The experiments examining the effects of Arf 5 and Rab35 silencing on cell migration support the conclusions made. The findings that Rab35 silencing causes upregulation of EGFR, leading to changes in expression of SPOCD1 is supported by the data. While more could be examined to probe the mechanism by which Arf5, Rab35 and downstream signals control gene expression to elicit changes in cell migration and tumor growth, in my view that is beyond the scope of this study. It appears that the authors are pursuing at least some such work based on the RNAseq data, which will be published as a separate study. In my view, this is appropriate.

3. There are no additional comments on this manuscript. This is an exciting and novel study.