Membrane association but not identity is required for LRRK2 activation and phosphorylation of Rab GTPases

Rachel Gomez, Paulina Wawro, Pawel Lis, Dario Alessi, and Suzanne Pfeffer

Corresponding Author(s): Suzanne Pfeffer, Stanford University School of Medicine

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Re: JCB manuscript #201902184

Dr. Suzanne R. Pfeffer
Stanford University School of Medicine
Department of Biochemistry
279 Campus Drive
Stanford, CA 94305-5307

Dear Dr. Pfeffer,

Thank you for submitting your manuscript entitled "Diffusion between membrane surfaces facilitates LRRK2 phosphorylation of Rab GTPases". 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 agree that your study contains potentially very interesting and novel findings regarding the mechanism of LRRK2 action. However, while they overall bring up similar concerns, they differ in the extent of experimental revisions required to address these issues, with reviewer #1 and #3 requiring further experimental support, while reviewer #2 suggests that they can be addressed mainly through extensive text edits. Overall, while the text changes suggested by reviewer #2 will all be quite useful in improving the accessibility of your finding to the readership of JCB, I agree that further experimental evidence is required to substantiate your main conclusions. However, experimentally testing if the phenotypes you describe are applicable to all pathogenic LRRK2 mutants (reviewer #1 point 2) is not required.

In particular:

- Further experimental evidence for your diffusion model is required, in particular the alternative experiments suggested by rev #1 point 10 and rev #3 point 1.
- Provide a better explanation for the use of your Rab mutants, as well as an experimental validation of their behavior (rev #1 point 3, rev #2 point 3, 4, rev#3 point 2, 4).
- Address the concerns regarding the use of lovastatin to assess prenylation (rev #1 p 4, rev #2 p 5, rev #3 p3).
- A better analysis and further controls for your colocalization experiments are required (rev #1 p5-10, rev #3 p 5, 6).

In addition, we hope that you will be able to address all of the remaining reviewer comments in your revised manuscript, including all issues regarding controls for expression levels, phosphorylation, quantification, and clarification to the materials and methods.

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.

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The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. 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 Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Harald Stenmark, PhD
Monitoring Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

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

The manuscript by Gomez and colleagues provides insights into the Rab29-mediated regulation of LRRK2 kinase activity, also in a subcellular context and with respect to the subsequent phosphorylation of the LRRK2 substrate Rab10. The authors show that Rab29 is poorly prenylated,

binds nucleotide weakly, and is not bound to GDI in the cytosol, yet only activates LRRK2 when membrane- and GTP-bound. When purposefully mis-targetted to mitochondria, Rab29 causes LRRK2 recruitment and substrate accumulation in this organelle, suggesting that LRRK2 can phosphorylate misdelivered Rab substrates which then become trapped in such subcellular compartment. In addition, the author´s data show that LRRK2 can phosphorylate Rab substrates in distinct membrane compartments by diffusing between such compartments, at least in the context of LRRK2 overexpression.

Whilst this manuscript contains a series of very interesting observations towards understanding how and where LRRK2 acts upon its substrates, I have several comments which need addressing:

Major:

1. Previous studies published by the authors suggest that the Rab29-mediated activation mechanism of LRRK2 involves altered phosphorylation of the N-terminal cellular phosphorylation sites within LRRK2 (e.g. Ser955 in R1441G LRRK2), mediated either by altered and/or phosphatase activities (Purlyte et al., 2018). This is an interesting hypothesis, and still valid in the light of the present data, especially if the (cytosolic) kinases and/or phosphatases responsible for the N-terminal LRRK2 phosphorylation status also diffuse between membranes, as they describe here for cytosolic LRRK2. Thus, the authors should determine the phosphorylation status of the distinct N-terminal residues in LRRK2 upon mito-Rab29 and R1441G LRRK2 co-expression, so as to confirm and/or refute whether altered LRRK2 phosphorylation at the N-terminal sites correlates with the Rab29-mediated activation mechanism also on mitochondrial membranes.

2. Are the phenotypes they describe applicable to all pathogenic LRRK2 mutants, including G2019S? The experiments should also be performed with this LRRK2 variant, as G2019S is the most prominent missense mutation in LRRK2 associated with Parkinson´s disease.

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The authors mention that release of MANT-GDP is two-fold faster for Rab29 as compared to Rab5 in the absence of EDTA. Please also show extended time courses to appreciate Rab5 off-rates over time.
They analyzed a Rab29 D63A mutant, and mention that they generated this mutant as binding to nucleotide extremely poorly, but then mention that it bound similar amounts of MANT-GDP compared with wild type Rab29, which seems contradictory (page 5). Please also show absolute nucleotide binding values for the experiments depicted in Figure 1, rather than normalizing all to 1 at time 0.
In addition, is the Rab29 D63A mutant still prenylated? They show that this mutant is cytosolic (Figure 3E), which would be consistent with a non-prenylated status of this protein. Perhaps the prenylation status influences nucleotide binding and/or nucleotide off-rates as assessed here? They should include a prenylation-deficient Rab29 construct in these assays, and attempt to compare prenylation status of wildtype Rab29, Rab29 D63A and prenylation-deficient C-terminal mutant, scaling up the assay as shown in Fig. 1F to also detect Rab29 prenylation status and differences between wildtype and those mutants, rather than just a comparative analysis of Rab10 versus Rab29 prenylation status. Also, quantifications (Figure 1D) are done over two experiments

performed in duplicate, which is not adequate. Please perform analyses over three experiments

4. Figure 2: The authors suggest that Rab29 is not GDI bound in the cytosol, yet its prenylation is needed for LRRK2 activation. Please show data of flag-LRRK2 transfected cells in the absence of myc-Rab29 co-transfection and in the absence or presence of lovastatin treatment, so as to assess whether lovastatin treatment per se has an effect on LRRK2 activity in the absence of Rab29 expression, and whether lovastatin treatment in the presence of myc-Rab29 reverts LRRK2 activity (assessed by S1292 phosphorylation) back to the levels as determined when only expressing LRRK2, or has additional effects. Also, please show phosphorylation of Rab10 substrate in those experiments to assess whether they track with LRRK2 activity as measured by S1292 phosphorylation.

5. Figure 3: The authors fused an amphipathic helix from MAO A to Rab29 to enrich mito-Rab29 on the outer membrane of mitochondria. As evidence, they show colocalization with mitofilin in HeLa cells.
It seems crucial to assure that this tagged, overexpressed mito-Rab29 protein only associates with mitochondria, rather than also associating with other membranes via such amphipathic helix. The authors should perform a proper colocalization analysis with various other markers (e.g. Golgi, recycling endosomes), as well as show the presence of mito-Rab29 (but not myc-Rab29) on purified mitochondrial fractions, and de-enrichment (or enrichment in the case of myc-Rab29) on other membranes. In such overexpression context, it is not sufficient to just show colocalization with mitofilin, and separation into cytosol/membrane fractions. Importantly, if a portion of overexpressed mito-Rab29 is localized to the same compartment as myc-Rab29 (Golgi), the same Golgi-mediated LRRK2 activation mechanism as previously reported may underlie the observations reported here. Also, in Figure 3B, please show expession levels of myc-Rab29 and mito-Rab29 in the same gel, so as to appreciate possible differences in expression and activation levels (since showing that both myc-Rab29 and mito-Rab29 display similar LRRK2 activation, Figure 3C). Please show tubulin as input in Figure 3B.

6. Figure 4: again, the reticular pattern of mito-Rab10 shown may reflect mitochondria, as well as other compartments. Please perform a careful colocalization and subcellular fractionation analysis to determine the precise localizations of overexpressed mito-Rab10 and myc-Rab10, and display in a quantitative manner. Also, display comparative quantifications of the phosphorylated levels of myc-Rab10 versus mito-Rab10 upon R1441G LRRK2 expression as shown by the representative panel (Figure 4B) (it is unclear whether similar levels of myc-Rab10 and mito-Rab10 are expressed to warrant direct comparisons; were these samples run in the same gels?). Figure 4B and 4C: please show tubulin as loading control.

7. Figure 5: please show Rab29-mediated recruitment, and quantification thereof, of R1441G LRRK2 to the Golgi apparatus as comparison (not shown for the R1441G mutant in Purlyte et al., 2018).
Importantly, the authors show that when relocalizing LRRK2 to mitochondria via expression of mito-Rab29, phosphorylated Rab10 also relocalizes to mitochondria. Careful colocalization analysis, and analysis of phosphorylated Rab10 levels on purified mitochondia is warranted here to corroborate this statement.
Their hypothesis is that Rab10 is promiscuously delivered to mitochondrial membranes in a GDI-dependent manner, but then cannot be extracted from those membranes upon LRRK2-mediated phosphorylation, as not able to interact with GDI when phosphorylated. Whilst an attractive hypothesis, how can the authors exclude that relocalization of phospho-Rab10 is not merely due to it binding to overexpressed LRRK2 without having been targetted to mitochondria in a GDI-

dependent manner, since substrates can bind to their kinases under such overexpression conditions? Can they biochemically determine differences in the levels of Rab10 in distinct subcellular compartments eg upon knockdown of GDI?
As evidence for their hypothesis, they show that Rab10 is retrieved from the membrane upon kinase inhibitor treatment (Fig. 5A). In this panel (+MLi2), they show LRRK2 colocalizing with mito-Rab29, yet their quantification (Figure 6) suggests a decrease in the interaction between LRRK2 and mito-Rab29 upon MLi2 treatment. Also, the MLi2 panel shows a very compact (Golgi?) mito-Rab29/LRRK2 localization. Are mitochondria and/or other structures affected upon MLi2 treatment? Again, colocalization analysis and mitochondrial purification approaches seem needed here to strengthen the conclusions. Also, staining with other markers to define the localization of endogenous Rab10 in the absence of LRRK2 expression, and in the absence or presence of MLi2 treatment, are warranted.
Figure 5B: please also show tubulin as loading control, and a quantification over several experiments to appreciate statistical significance. The legend to the figure states that MLi2 treatment was performed at the time of transfection. Please use similar short-term MLi2 treatment shown to relocalize Rab10 as employed for Figure 5A (eg. 45 minutes).

8. Figure 6: Again, scoring "mitochondrial appearance" is not sufficient to assure that the structures are mitochondria. Stain for a mitochondrial marker in red to perform such quantifications. Also, use R1441G-D2017A mutant (employed in Figure 2), rather than just D2017A, as a better control for these experiments in the context of modulating the kinase activity of R1441G.
The authors mention that Rab29 is more able to recruit LRRK2 to mitochondria as compared to inactive Rab29 D63A, despite similar expression levels (page 11). Please show expression level data.
Also, they mention: "Rab29 phosphorylation after LRRK2 activation may turn off this activation pathway" (page 11). In the absence of data shown to support this statement, it seems best to delete this phrase.

9. Figure 7: again, colocalization studies with additional markers, as well as subcellular fractionation to assess the pool of overexpressed myc-Rab10 and mito-LRRK2, and levels of phospho-Rab10 in distinct fractions (e.g. Golgi versus mitochondria) is necessary to corroborate conclusions regarding localization. Also, please show reversal of phospho-Rab10 staining by MLi2, and show quantification of the observations (as done for Figure 6).

10. Figure 8: Please show additional evidence that PM-Rab10 is plasma membrane localized, rather than additionally localized to other intracellular compartments. Also, panel A, please show tubulin input. They again mention in the legend that MLi2 was added at the time of transfection. Please repeat with shorter incubation of inhibitor.
The data presented here, namely that plasma membrane-targetted Rab10 is also phosphorylated by soluble LRRK2, but not by mitochondrially targetted LRRK2, is somewhat not surprising, as one could expect that a cytosolic kinase can "diffuse" to phosphorylate its substrates which are localized at distinct intracellular cytosol-accessible locations. Perhaps a better experiment to test their more important hypothesis, namely that membrane-targetted LRRK2 can "trap" mislocalized Rab10 in distinct intracellular membranes, would be to target LRRK2 to the plasma membrane, and then determine whether it also causes aberrant accumulation of phospho-Rab10 in the plasma membrane, as shown when artificially targetting LRRK2 to mitochondria. This would also help extend the surprising observation that membrane-targetted LRRK2 is more active (as assessed by 1292 phosphorylation), yet causes comparable substrate phosphorylation.
Figure 8B: please also show localization of GFP-R1441G LRRK2.

Figure 8D: the y axis label should read "1292 LRRK2 phosphorylation", not "wildtype myc-Rab10 phosphorylation", as referred to in the text (page 14). Also please amend Figure legend accordingly.

Minor:

Materials and Methods (pages 18 and following):
1. please specify species of construct sequences employed (all human?)
2. please provide reference describing that amphipathic helix from MAO A exclusively targets to mitochondria.
3. please provide details (company, order number) for phospho-Rab10 antibodies employed for Western blotting, as well as for immunocytochemistry purposes.
4. page 21: 120 µg of lysate analysed by immunoblot?
5. Please provide additional details of confocal settings employed, and quantifications performed.

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

The discovery that LRRK2 phosphorylates Rab proteins has focused the attention on the regulation of this mechanism with respect to Parkinson's disease. This manuscript presents some careful work toward elucidating the mechanism of LRRK2 mediated phosphorylation of its Rab substrates, especially highlighting the role of Rab29 in modulating these events. This work has some very interesting conclusions drawn on a series of ingenious and intriguing experiments. One is that LRRK2 fulfils the criteria of a Rab29 effector. However, Rab29 has an unusual behavior in that it binds nucleotide poorly, is not efficiently prenylated, and not complexed to GDI in cytosol. Despite being both Rab29 and LRRK2 being cytosolic, Rab29 needs to be membrane localized and GTP-bound to activate LRRK2 and have it to phosphorylate Rabs. However, the targeting of Rab29 to the mitochondrial surface suggests that a (Golgi) specific membrane localization is per se not required, which is surprising, and lead to the accumulation of pRab10 on mitochondria because it cannot be extracted by GDI. These results have implications for the general mechanisms regulating the correct intracellular distribution of Rab proteins.

This is a very interesting body of work and should be published once these relatively minor changes are completed. In general, the results are nice and address an important regulatory mechanism. The writing however could be much improved as, in general, the topics are not sufficiently well introduced and a reader not well versed in both Rabs and LRRK2 will struggle. Therefore, most of my comments can be addressed by reorganizing the text.

Specific comments
1. There are a number of points which are developed in the Results but need to be addressed already in the Introduction to better explain the knowledge base for LRRK2 activation and the role for Rab GTPases: 1) how is LRRK2 localized to membranes, 2) how is LRRK2 activated, and 3) is Rab29 the only Rab linked to LRRK2 activation? Other points which are not clearly introduced or explained are: why other Rabs are chosen as specific controls in various experiments. Lastly, a point of confusion is the difference between membrane diffusion and cytosolic diffusion of LRRK2, including in the title of the manuscript. This work has some very interesting conclusions but I am not sure that the peculiar features of Rab29 are sufficiently important or explained to be appreciated in

the abstract.

2. The R1441G mutant of LRRK2 is used throughout the manuscript. Please indicate why the WT protein was not used and compare how localization of this mutant might differ from WT protein (page 3). Also on page 3, please remove the sentence stating that the switch regions of Rabs are the only regions altered by nucleotide binding. This is misleading as those studies were completed with truncated proteins. More recent molecular dynamics simulations using full-length proteins predict more extensive involvement.

3. The Rab29 mutants V156A and I64T were used for nucleotide release experiments and nothing further (Figure 1B, 1D). I can't see which useful insight they provide. Also, the D63A mutant should be described as being created to offer a tool with which to separate nucleotide binding from membrane association (along with other mutants), rather than being merely a "non-functional mutant". Also, the non-normalized nucleotide release data should be provided as a supplementary figure. That will give better insight into relative nucleotide binding efficiency and is necessary for interpretation of the data.

4. The section "Cytosolic Rab29 is not GDI-bound" is awkwardly written. The conclusion is important, but the usage of words is imprecise. For example "Rab29 chromatographs as a monomer" is inaccurate and misleading as it may suggest that Rab29 could be a dimer, rather than suggesting that it could be a Rab29:GDI complex. The important point is that insufficiently prenylated Rabs will have difficulty associating with membranes and with GDI because both interactions are mediated by the lipid. Also careful with the sentence stating that prenylation is dependent on GDP binding, as it is based on biochemistry assays comparing GDP and GTP/GTP analogs.

5. I would be careful with the interpretation of the lovastatin results (Figure 2A, B): Interfering broadly with prenylation alters many small GTPases, not even just Rabs and not only Rab29. This heavy handed approach is unnecessary as later experiments targeting only Rab29 prenylation allow for the same conclusion with a more elegant approach. I recommend to add a quantification of co-localization to Figure 3A. Figure 4C is not convincing and should either be removed or repeated with cleaner results. I would combine Figures 5 and 6 and add a quantification of the co-localization data in Figure 5. Also, please add a panel showing the pRab10 staining in the presence of MLi2.

6. The conclusion of the second paragraph on page 10 regarding the role of GDI binding and Rab phosphorylation being intimately connected to LRRK2 activity is important and deserves more attention. This concept is touched upon again at the end of page 11 and should be strengthened in the discussion.

7. More on Results and Discussion. The first sentence on page 12 regarding Rab localization to the Golgi (or lack thereof) requires a reference or illustrative data. Also, please address why the Rab substrates are localized to mitochondria when LRRK2 is forced onto the mitochondria, but the same does not happen when LRRK2 is localized to the Golgi under normal conditions. The first sentence on page 13 also requires a reference to a figure containing the relevant data.
The last sentence of the results section is an important discussion point. Does membrane binding or activation of LRRK2 induce or stabilize a dimer/monomer transition? There is precedent in bacterial Roco proteins showing dramatic conformational changes upon nucleotide binding and could be used to support some suggested regulatory mechanisms here. Another place for discussion is the curious nucleotide binding and prenylation differences in Rab29 compared with other Rabs. Is there a sequence homology or structural explanation? Would you expect Rab29 to

be the only protein with these behaviors?

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

Mutations in Leucine-rich-repeat-kinase 2 (LRRK2), the causative gene for PARK8, are the most prevalent genetic causes of familial Parkinson's disease (PD). Determination of the regulatory mechanism of LRRK2 kinase activity is an important clue to understanding the pathogenesis of PD. Recently, several groups, including authors' group, have identified a subset of Rab GTPases as LRRK2 substrates, and Rab29 has been shown to activate LRRK2 and recruit it to the Golgi followed by Rab10 phosphorylation there. In this manuscript, the authors extended their previous findings and showed that Rab29 is an atypical Rab that is poorly prenylated and is not bound to GDI in the cytosol. They then showed that plasma membrane-anchored Rab10 is poorly phosphorylated by ectopically anchored LRRK2 on mitochondria (mito-LRRK2) when compared to soluble LRRK2, whereas mito-LRRK2 can phosphorylate wild-type Rab10. Finally, the authors proposed a novel model in which LRRK2 diffuses between membranes to phosphorylate Rab substrates. Although the diffusion model for LRRK2 kinase action is fascinating, the present data are insufficient to support this model. Additional experiments are necessary to strengthen the authors' conclusions.

Specific points:
1. As noted above, the diffusion model should be strengthened experimentally. I suggest the authors to perform live cell imaging by using photoactivatable or photoconvertible fluorescent proteins. Furthermore, it should also be clarified whether activated LRRK2 is still associated with Rab29 and, if not, what other factors help LRRK2 to be retained on the membrane? Such a mechanistic insight is needed to explain how LRRK2 diffuses to distant membranes, keeping associated with the membrane rather than released into the cytosol.

2. In Figure 1, the authors suggested that canonical mutations should not be used to study Rab29. Indeed, the Q67L mutant of Rab29 does not behave as a constitutively active mutant; however, there seems no reason to avoid using the T21N mutant as an unfunctional Rab29. What is the advantage of V156A, I64T, and D63A mutants when compared to the T21N mutant? Comparing binding/releasing rate of GDP and GTP, prenylation efficiency among these mutants would reveal the difference between them. I also wondered whether "endogenous Rab29" actually loses GDI binding and is poorly prenylated unlike other Rabs because the authors only used overexpressed, recombinant Rab29 proteins throughout this study.

3. Since lovastatin inhibits prenylation of all Rabs, it is important to test whether the observed effect on LRRK2 activation (Figure 2A) is directly related to inability to prenylate Rab29. The authors should test the effect of lovastatin on LRRK2 activation in the absence of Myc-Rab29 as a negative control.

4. The authors prepared and used Rab29 D63A as a cytosolic-localized mutant, but it is not clear whether this mutant still functions as a Rab29 effector, especially when it is anchored to mitochondria (Figure 3). Co-IP experiments should be performed to test the interaction between LRRK2 and Rab29 WT or D63A.

5. Co-localization of LRRK2 and Rab10 in mito-Rab29-expressing cells is not clear at all (Figure 5A).

The authors should stain the cells with appropriate organelle markers (Golgi and mitochondria) and quantify co-localization rates between Rab10 (or LRRK2) and each marker. Also, the phosphorylation rate of Rab10 should be evaluated by using the Phos-tag-based immunoblotting (see the authors' previous papers; eLife 2017;6:e31012 and Biochem. J. 2016;473:2671-2685) (Figure 5B).

6. It is not clear how the authors quantify the percentage of cells with LRRK2 on mitochondria in Figure 6A. The authors need to describe the detailed method and add negative control data (i.e., without mito-Rab29). Also, typical images of cells in each sample should be provided in Figure 6B.

(Other minor points)
7. MLi-2 treatment alone seemed to disrupt mitochondrial morphology and distribution in Figure 5. Is it true?

8. For most of the immunoblot data, it is not clear which antibodies were used to detect immunoreactive bands. For example, was Myc-Rab29 detected with anti-Myc antibody or anti-Rab29 antibody in Figure 2A? The authors should include more detailed information in each figure legend. Also, addition of lane numbers to immunoblot data is helpful for general readers.

Response to Editor and Reviewer Comments

We are grateful to the reviewers for their thoughtful comments; we have provided almost all of the additional data and quantification requested and hope that the revised manuscript will now be deemed suitable for presentation in J. Cell Biol.

Editor's Comments:

- Further experimental evidence for your diffusion model is required, in particular the alternative experiments suggested by rev #1 point 10 and rev #3 point 1. **We did the Rev #1 point 10 experiment but Rev #3 point 1 cannot be done because 80% of the kinase is in cytosol and will mask movement of kinase from one membrane surface to another**
- Provide a better explanation for the use of your Rab mutants, as well as an experimental validation of their behavior (rev #1 point 3, rev #2 point 3, 4, rev#3 point 2, 4). **We removed two mutants, included better description of D63A, and provided the additional time courses and uncorrected graphs as requested**
- Address the concerns regarding the use of lovastatin to assess prenylation (rev #1 p 4, rev #2 p 5, rev #3 p3). **We agree completely with the reviewer and softened the conclusions; we also added a control to show that lovastatin alone does not influence LRRK2 activation monitored with anti-pS1292 antibody.**
- A better analysis and further controls for your colocalization experiments are required (rev #1 p5-10, rev #3 p 5, 6). **We thank the reviewers for this and provide a new Fig 3 with multiple markers as well as Pearson's correlation coefficients for all localizations throughout**

In addition, we hope that you will be able to address all of the remaining reviewer comments in your revised manuscript, including all issues regarding controls for expression levels, phosphorylation, quantification, and clarification to the materials and methods. **See above and also, we now provide loading control Ponceau stains or other markers in addition to normalizing to the LRRK2 levels that we believe are most informative.**

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.

SPECIFIC RESPONSES TO REVIEWER COMMENTS

Reviewer #1

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Purlyte et al. showed increased phosphorylation of S955 but not S935 upon expression of Rab29. We don't yet fully understand these distinct sites and the kinases responsible for their modifications, and for these reasons, feel that while this is an interesting point, it is beyond the scope of the present study.

2. Are the phenotypes they describe applicable to all pathogenic LRRK2 mutants, including G2019S? The experiments should also be performed with this LRRK2 variant, as G2019S is the most prominent missense mutation in LRRK2 associated with Parkinson´s disease.

G2019S is also activated and recruited to the Golgi by Rab29 (Purlyte et al.) so we have every reason to believe that it would be recruited to mitochondria by Rab29. We thank the Editor for understanding that this is not necessary to support our conclusions.

3. Figure 1: what was the rationale for generating the V156A and I64T mutants in Rab29? The rationale for their design, either based on previous studies with other Rabs along with relevant citations, and/or structurally guided approaches for generating these mutants are required here. Are these mutants cytosolic? Are they still prenylated? Are they still able to bind and thus recruit LRRK2? What is their effect on the distinct assays they show?

We have removed all mention of V156A and I64T mutants of Rab29 from this paper to avoid any confusion.

The authors mention that release of MANT-GDP is two-fold faster for Rab29 as compared to Rab5 in the absence of EDTA. Please also show extended time courses to appreciate Rab5 off-rates over time.

As requested, we now include the extended time courses for both MANT-GDP experiments in 1B/1D.
They analyzed a Rab29 D63A mutant, and mention that they generated this mutant as binding to nucleotide extremely poorly, but then mention that it bound similar amounts of MANT-GDP compared with wild type Rab29, which seems contradictory (page 5).

When two proteins with different binding affinities are provided nucleotide far above their K_Ds, they will both acquire nucleotide but the weaker binding protein will release it faster. We modified the text to make this clearer for the reader.

Please also show absolute nucleotide binding values for the experiments depicted in Figure 1, rather than normalizing all to 1 at time 0.

As requested, Figures 1B and 1D now show raw values as well.

In addition, is the Rab29 D63A mutant still prenylated? They show that this mutant is cytosolic (Figure 3E), which would be consistent with a non-prenylated status of this protein. Perhaps the prenylation status influences nucleotide binding and/or nucleotide off-rates as assessed here? They should include a prenylation-deficient Rab29 construct in these assays and attempt to compare prenylation status of wildtype Rab29, Rab29 D63A and prenylation-deficient C-terminal mutant, scaling up the assay as shown in Fig. 1F to also detect Rab29 prenylation status and differences between wildtype and those mutants, rather than just a comparative analysis of Rab10 versus Rab29 prenylation status.

This binding assays in Fig. 1 were performed with bacterially-derived protein: in these experiments, neither WT nor D63A are prenylated. In previous work we have shown at least for Rab9, that prenylation has no effect on nucleotide binding affinity (Shapiro and Pfeffer, 1993). In cells, the protein is likely not prenylated because the prenylation machinery is known to care about Rab-nucleotide binding.

We only conclude that the protein is less efficiently prenylated, and that is a reasonable explanation for why it fails to bind GDI. We agree with the reviewer that additional experiments would enable us to provide precise details related to this issue, but that is not the main focus of the paper and would distract us from the most important aspects. Thus, we prefer to pursue that independent of this story.

Also, quantifications (Figure 1D) are done over two experiments performed in duplicate, which is not adequate. Please perform analyses over three experiments

Figure 1E: we have included another experiment so analysis is over 3 experiments.

4. Figure 2: The authors suggest that Rab29 is not GDI bound in the cytosol, yet its prenylation is needed for LRRK2 activation. Please show data of flag-LRRK2 transfected cells in the absence of myc-Rab29 co-transfection and in the absence or presence of lovastatin treatment, so as to assess whether lovastatin treatment per se has an effect on LRRK2 activity in the absence of Rab29 expression, and whether

lovastatin treatment in the presence of myc-Rab29 reverts LRRK2 activity (assessed by S1292 phosphorylation) back to the levels as determined when only expressing LRRK2, or has additional effects. Also, please show phosphorylation of Rab10 substrate in those experiments to assess whether they track with LRRK2 activity as measured by S1292 phosphorylation.

We now show in 2A and 2B that lovastatin alone does not influence pS1292 levels and the activation seen with Rab29 is eliminated upon lovastatin treatment. We showed previously (Purlyte et al.) that pRab10 levels track with Rab29 activation of LRRK2.

5. Figure 3: The authors fused an amphipathic helix from MAO A to Rab29 to enrich mito-Rab29 on the outer membrane of mitochondria. As evidence, they show colocalization with mitofilin in HeLa cells. It seems crucial to assure that this tagged, overexpressed mito-Rab29 protein only associates with mitochondria, rather than also associating with other membranes via such amphipathic helix. The authors should perform a proper colocalization analysis with various other markers (e.g. Golgi, recycling endosomes), Also, in Figure 3B, please show expression levels of myc-Rab29 and mito-Rab29 in the same gel, so as to appreciate possible differences in expression and activation levels (since showing that both myc-Rab29 and mito-Rab29 display similar LRRK2 activation, Figure 3C). Please show tubulin as input in Figure 3B.

We thank the reviewer for this important comment and have now added a new figure 3, which shows lack of colocalization with Golgi, early endosomes, lysosomes, and endoplasmic reticulum. Figure 4 shows Pearson's correlation coefficient between mito-Rab29 and those compartments in two cell types (HeLa and RPE cells). Myc-Rab29 and mito-Rab29 are on the same gel in figure 4B. We also show a loading control.

as well as show the presence of mito-Rab29 (but not myc-Rab29) on purified mitochondrial fractions, and de-enrichment (or enrichment in the case of myc-Rab29) on other membranes.

We do not believe that fractionation of cells is more accurate than the careful and now quantitative light microscopy shown here, as mitochondrially enriched fractions may contain contaminating membranes of other types. We also added plasma membrane targeted proteins to this revised manuscript and obtain similar findings, independently substantiating our approach.

6. Figure 4: again, the reticular pattern of mito-Rab10 shown may reflect mitochondria, as well as other compartments. Please perform a careful colocalization and subcellular fractionation analysis to determine the precise localizations of overexpressed mito-Rab10 and myc-Rab10, and display in a quantitative manner.

We have now included the Pearson's correlation coefficient between mito-Rab10 and the mitochondrial marker mitofilin and at 0.81, confirms excellent co-localization.

Also, display comparative quantifications of the phosphorylated levels of myc-Rab10 versus mito-Rab10 upon R1441G LRRK2 expression as shown by the representative panel (Figure 4B) (it is unclear whether similar levels of myc-Rab10 and mito-Rab10 are expressed to warrant direct comparisons; were these samples run in the same gels?). Figure 4B and 4C: please show tubulin as loading control.

We have added quantifications of mito Rab phosphorylation in new figure 5D and 5E; in Fig. 4 (now Fig. 5), LRRK2 is our loading (and normalization) control and Ponceau S stains confirm equal protein loading.

7. Figure 5: please show Rab29-mediated recruitment, and quantification thereof, of R1441G LRRK2 to the Golgi apparatus as comparison (not shown for the R1441G mutant in Purlyte et al., 2018).

See top of new Fig. 6 for colocalization of R1441G LRRK2, Rab29, and GCC185. Quantitation has also been shown by Madero Perez et. al. (2018).

Importantly, the authors show that when relocalizing LRRK2 to mitochondria via expression of mito-Rab29, phosphorylated Rab10 also relocalizes to mitochondria. Careful colocalization analysis, and analysis of phosphorylated Rab10 levels on purified mitochondria is warranted here to corroborate this statement.

Detection of phosphorylated Rab10 (especially endogenous) is not easy, and detecting it in purified mitochondrial fractions would take much more material. More importantly, some amount of dephosphorylation during sample processing is bound to occur making it more difficult to detect. We have now included Pearson's correlation coefficients for colocalization between LRRK2 and endogenous pRab10 in new Fig. 7A; the quantitation supports our conclusions.

Their hypothesis is that Rab10 is promiscuously delivered to mitochondrial membranes in a GDI-dependent manner, but then cannot be extracted from those membranes upon LRRK2-mediated phosphorylation, as not able to interact with GDI when phosphorylated. Whilst an attractive hypothesis, how can the authors exclude that relocalization of phospho-Rab10 is not merely due to it binding to overexpressed LRRK2 without having been targetted to mitochondria in a GDI-dependent manner, since substrates can bind to their kinases under such overexpression conditions? Can they biochemically determine differences in the levels of Rab10 in distinct subcellular compartments eg upon knockdown of GDI?

Rab10 is fully prenylated (two 20 carbon branched hydrocarbon tails), thus is entirely stable on membranes and does not move without GDI. GDI depletion kills cells. Also, we now show that kinase dead D2017A mito-LRRK2 recruits much less Rab10 as determined by Pearson's correlation coefficient (new Fig 7A). Thus, the relocalized kinase is not sufficient to explain relocalization of Rab10 to mitochondria.

As evidence for their hypothesis, they show that Rab10 is retrieved from the membrane upon kinase inhibitor treatment (Fig. 5A). In this panel (+MLi2), they show LRRK2 colocalizing with mito-Rab29, yet their quantification (Figure 6) suggests a decrease in the interaction between LRRK2 and mito-Rab29 upon MLi2 treatment.

Careful quantification in new figure 7A actually shows that LRRK2 activity does not influence its ability to bind to Rab29.

Also, the MLi2 panel shows a very compact (Golgi?) mito-Rab29/LRRK2 localization. Are mitochondria and/or other structures affected upon MLi2 treatment?

No, please see the other example in figure 6A third row.

Again, colocalization analysis and mitochondrial purification approaches seem needed here to strengthen the conclusions. Also, staining with other markers to define the localization of endogenous Rab10 in the absence of LRRK2 expression, and in the absence or presence of MLi2 treatment, are warranted.

Co-localization is now shown in new Fig. 6A, fourth panel and quantified in Fig. 7A.

Figure 5B: please also show tubulin as loading control, and a quantification over several experiments to appreciate statistical significance.

We have added Ponceau stains or other loading markers for many of the blots as requested. This panel is shown to simply validate MLi-2 inhibition of Rab10 phosphorylation, detected by IF, as is now well established.

The legend to the figure states that MLi2 treatment was performed at the time of transfection. Please use similar short-term MLi2 treatment shown to relocalize Rab10 as employed for Figure 5A (eg. 45 minutes).
It is not clear to us why this is important; it is well established that MLi-2 blocks LRRK2 within 45 minutes by western blot (For example, Ito et. al 2016).

8. Figure 6: Again, scoring "mitochondrial appearance" is not sufficient to assure that the structures are mitochondria. Stain for a mitochondrial marker in red to perform such quantifications. Also, use R1441G-D2017A mutant (employed in Figure 2), rather than just D2017A, as a better control for these experiments in the context of modulating the kinase activity of R1441G.

Our careful analysis has shown that D2017A LRRK2 is recruited by Rab29 similar to R1441G LRRK2 (new Fig. 7A) so the double mutant is not necessary

The authors mention that Rab29 is more able to recruit LRRK2 to mitochondria as compared to inactive Rab29 D63A, despite similar expression levels (page 11). Please show expression level data.

This can be found in the new Fig. 4B and 4E

Also, they mention: "Rab29 phosphorylation after LRRK2 activation may turn off this activation pathway" (page 11). In the absence of data shown to support this statement, it seems best to delete this phrase.

Is it not ok to speculate in the discussion to guide future experiments? We believe it's ok and actually important.
9. Figure 7: again, colocalization studies with additional markers, as well as subcellular fractionation to assess the pool of overexpressed myc-Rab10 and mito-LRRK2, and levels of phospho-Rab10 in distinct fractions (e.g. Golgi versus mitochondria) is necessary to corroborate conclusions regarding localization.

As described above, we feel strongly that cell fractionation is less strong than moving LRRK2 to the plasma membrane as an additional and alternative location and seeing the same phenomena at a different location.

Also, please show reversal of phospho-Rab10 staining by MLi2, and show quantification of the observations (as done for Figure 6).
See new Fig. 6 row 3

10. Figure 8: Please show additional evidence that PM-Rab10 is plasma membrane localized, rather than additionally localized to other intracellular compartments.

Given the general staining of the plasma membrane, one cannot rule out that a small fraction is on another compartment. However, the entire confocal Z stack is shown and the appearance of cytoplasmic LRRK2 is quite different from that of PM-Rab10. We have added this caution to the text as not to mislead the reader.

Also, panel A, please show tubulin input. **Loading control added as requested, although it is most important to normalize to LRRK2 levels.** They again mention in the legend that MLi2 was added at the time of transfection. Please repeat with shorter incubation of inhibitor.

We do not see the importance of shorter MLi treatment in this experiment; it was carried out simply to verify that the PM-pRab10 seen by IF is sensitive to LRRK2 inhibition. The conclusions of this experiment do not rely on this aspect.

The data presented here, namely that plasma membrane-targetted Rab10 is also phosphorylated by soluble LRRK2, but not by mitochondrially targetted LRRK2, is somewhat not surprising, as one could expect that a cytosolic kinase can "diffuse" to phosphorylate its substrates which are localized at distinct intracellular cytosol-accessible locations. Perhaps a better experiment to test their more important hypothesis, namely that membrane-targetted LRRK2 can "trap" mislocalized Rab10 in distinct intracellular membranes, would be to target LRRK2 to the plasma membrane, and then determine whether it also causes aberrant accumulation of phospho-Rab10 in the plasma membrane, as shown when artificially targetting LRRK2 to mitochondria. This would also help extend the surprising observation that membrane-targetted LRRK2 is more active (as assessed by 1292 phosphorylation), yet causes comparable substrate phosphorylation.

We have carried out the requested experiment--See figure 8 bottom panel and Fig. 7A.

Figure 8B: please also show localization of GFP-R1441G LRRK2.

Now shown in Fig. 9B

Figure 8D: the y axis label should read "1292 LRRK2 phosphorylation", not "wildtype myc-Rab10 phosphorylation", as referred to in the text (page 14). Also please amend Figure legend accordingly.
We have changed the manner of normalization of new figure 9C and D and have amended the figure legend accordingly.
Minor: Materials and Methods (pages 18 and following):
1. please specify species of construct sequences employed (all human?)

We now specify that all of the construct sequences employed are human.
2. please provide reference describing that amphipathic helix from MAO A exclusively targets to mitochondria. **Please see figure 3. For another example of use of this tag, see Wong, M., Munro S. 2014. The specificity of vesicle traffic to the Golgi is encoded in the Golgin coiled-coil proteins. *Science*. 346: 6209.**

3. please provide details (company, order number) for phospho-Rab10 antibodies employed for Western blotting, as well as for immunocytochemistry purposes.

This has been clarified in the materials and methods section.

4. page 21: 120 µg of lysate analysed by immunoblot?

That is correct

5. Please provide additional details of confocal settings employed, and quantifications performed.

We have added typical exposure settings.

Reviewer #2 The discovery that LRRK2 phosphorylates Rab proteins has focused the attention on the regulation of this mechanism with respect to Parkinson's disease. This manuscript presents some careful work toward elucidating the mechanism of LRRK2 mediated phosphorylation of its Rab substrates, especially highlighting the role of Rab29 in modulating these events. This work has some very interesting conclusions drawn on a series of ingenious and intriguing experiments. One is that LRRK2 fulfils the criteria of a Rab29 effector. However, Rab29 has an unusual behavior in that it binds nucleotide poorly, is not efficiently prenylated, and not complexed to GDI in cytosol. Despite being both Rab29 and LRRK2 being cytosolic, Rab29 needs to be membrane localized and GTP-bound to activate LRRK2 and have it to phosphorylate Rabs. However, the targeting of Rab29 to the mitochondrial surface suggests that a (Golgi) specific membrane localization is per se not required, which is surprising, and lead to the accumulation of pRab10 on mitochondria because it cannot be extracted by GDI. These results have implications for the general mechanisms regulating the correct intracellular distribution of Rab proteins.

This is a very interesting body of work and should be published once these relatively minor changes are completed. In general, the results are nice and address an important regulatory mechanism. The writing however could be much improved as, in general, the topics are not sufficiently well introduced and a reader not well versed in both Rabs and LRRK2 will struggle. Therefore, most of my comments can be addressed by reorganizing the text**. [WE THANK THE REFEREE FOR THEIR POSITIVE ASSESSMENT]**

Specific comments

1. There are a number of points which are developed in the Results but need to be addressed already in the Introduction to better explain the knowledge base for LRRK2 activation and the role for Rab GTPases: 1) how is LRRK2 localized to membranes, **[NOT KNOWN]** 2) how is LRRK2 activated**, [NOT KNOWN]** and 3) is Rab29 the only Rab linked to LRRK2 activation? **[YES AT THIS POINT IN TIME]** Other points which are not clearly introduced or explained are: why other Rabs are chosen as specific controls in various experiments. Lastly, a point of confusion is the difference between membrane diffusion and cytosolic diffusion of LRRK2, including in the title of the manuscript **[THANK YOU for catching this--we have changed the title]**. This work has some very interesting conclusions but I am not sure that the peculiar features of Rab29 are sufficiently important or explained to be appreciated in the abstract.

We have modified the text accordingly to try to address these issues.

2. The R1441G mutant of LRRK2 is used throughout the manuscript. Please indicate why the WT protein was not used and compare how localization of this mutant might differ from WT protein (page 3).

We have tried to clarify the text regarding this issue

Also on page 3, please remove the sentence stating that the switch regions of Rabs are the only regions altered by nucleotide binding. This is misleading as those studies were completed with truncated proteins. More recent molecular dynamics simulations using full-length proteins predict more extensive

involvement.

Sentence modified as requested

3. The Rab29 mutants V156A and I64T were used for nucleotide release experiments and nothing further (Figure 1B, 1D). I can't see which useful insight they provide. Also, the D63A mutant should be described as being created to offer a tool with which to separate nucleotide binding from membrane association (along with other mutants), rather than being merely a "non-functional mutant". Also, the non-normalized nucleotide release data should be provided as a supplementary figure. That will give better insight into relative nucleotide binding efficiency and is necessary for interpretation of the data.

We have removed V156A and I64T data from the manuscript, modified the text and included the non-normalized data and longer time courses in Figs. 1B and D

4. The section "Cytosolic Rab29 is not GDI-bound" is awkwardly written. The conclusion is important, but the usage of words is imprecise. For example "Rab29 chromatographs as a monomer" is inaccurate and misleading as it may suggest that Rab29 could be a dimer, rather than suggesting that it could be a Rab29:GDI complex. The important point is that insufficiently prenylated Rabs will have difficulty associating with membranes and with GDI because both interactions are mediated by the lipid. Also careful with the sentence stating that prenylation is dependent on GDP binding, as it is based on biochemistry assays comparing GDP and GTP/GTP analogs.

We have rewritten this paragraph as requested

5. I would be careful with the interpretation of the lovastatin results (Figure 2A, B): Interfering broadly with prenylation alters many small GTPases, not even just Rabs and not only Rab29. This heavy handed approach is unnecessary as later experiments targeting only Rab29 prenylation allow for the same conclusion with a more elegant approach.

We agree and modified the text accordingly.

I recommend to add a quantification of co-localization to Figure 3A.

Added quantification of co-localization and additional markers in new figures 3 and 4A

Figure 4C is not convincing and should either be removed or repeated with cleaner results.

As requested, blot replaced in (now) figure 5C and quantified in figure 5E.

I would combine Figures 5 and 6 and add a quantification of the co-localization data in Figure 5. Also, please add a panel showing the pRab10 staining in the presence of MLi2.

Added quantification of colocalization between 1441G LRRK2 and pRab10 in (now) figure 7A and added a pRab10 staining in presence of MLi-2 (now figure 6A third panel).

6. The conclusion of the second paragraph on page 10 regarding the role of GDI binding and Rab phosphorylation being intimately connected to LRRK2 activity is important and deserves more attention. This concept is touched upon again at the end of page 11 and should be strengthened in the discussion.

Amplified on page 17

7. More on Results and Discussion. The first sentence on page 12 regarding Rab localization to the Golgi (or lack thereof) requires a reference or illustrative data.

We have corrected the text as requested

Also, please address why the Rab substrates are localized to mitochondria when LRRK2 is forced onto the mitochondria, but the same does not happen when LRRK2 is localized to the Golgi under normal conditions.

We have clarified the text accordingly

The first sentence on page 13 also requires a reference to a figure containing the relevant data.

Corrected as requested

The last sentence of the results section is an important discussion point. Does membrane binding or activation of LRRK2 induce or stabilize a dimer/monomer transition? There is precedent in bacterial Roco proteins showing dramatic conformational changes upon nucleotide binding and could be used to support some suggested regulatory mechanisms here. Another place for discussion is the curious nucleotide binding and prenylation differences in Rab29 compared with other Rabs. Is there a sequence homology or structural explanation? Would you expect Rab29 to be the only protein with these behaviors?

We have expanded the discussion as requested

Reviewer #3 Mutations in Leucine-rich-repeat-kinase 2 (LRRK2), the causative gene for PARK8, are the most prevalent genetic causes of familial Parkinson's disease (PD). Determination of the regulatory mechanism of LRRK2 kinase activity is an important clue to understanding the pathogenesis of PD. Recently, several groups, including authors' group, have identified a subset of Rab GTPases as LRRK2 substrates, and Rab29 has been shown to activate LRRK2 and recruit it to the Golgi followed by Rab10 phosphorylation there. In this manuscript, the authors extended their previous findings and showed that Rab29 is an atypical Rab that is poorly prenylated and is not bound to GDI in the cytosol. They then showed that plasma membrane-anchored Rab10 is poorly phosphorylated by ectopically anchored LRRK2 on mitochondria (mito-LRRK2) when compared to soluble LRRK2, whereas mito-LRRK2 can phosphorylate wild-type Rab10. Finally, the authors proposed a novel model in which LRRK2 diffuses between membranes to phosphorylate Rab substrates. Although the diffusion model for LRRK2 kinase action is fascinating, the present data are insufficient to support this model. Additional experiments are necessary to strengthen the authors' conclusions.

Specific points:

1. As noted above, the diffusion model should be strengthened experimentally. I suggest the authors to perform live cell imaging by using photoactivatable or photoconvertible fluorescent proteins. Furthermore, it should also be clarified whether activated LRRK2 is still associated with Rab29 and, if not, what other factors help LRRK2 to be retained on the membrane? Such a mechanistic insight is needed to explain how LRRK2 diffuses to distant membranes, keeping associated with the membrane rather than released into the cytosol.

Unfortunately 80% of LRRK2 is cytosolic and would mask any diffusion of a membrane associated protein upon live cell imaging. We have clarified the text to explain that even if we had anchored Rab29 and Rab10 permanently on different membranes, we would not be able to know if LRRK2 required Rab29 binding for maintenance of a hyper-activated state as LRRK2 can phosphorylate substrates even in cells lacking Rab29 protein.

2. In Figure 1, the authors suggested that canonical mutations should not be used to study Rab29. Indeed, the Q67L mutant of Rab29 does not behave as a constitutively active mutant; however, there seems no reason to avoid using the T21N mutant as an unfunctional Rab29. What is the advantage of V156A, I64T, and D63A mutants when compared to the T21N mutant?

We removed V156A and I64T mutants. In our hands and in others (cited) Rab29 T21N is not completely cytosolic: some still makes it to the Golgi.

Comparing binding/releasing rate of GDP and GTP, prenylation efficiency among these mutants would reveal the difference between them.

We have removed these mutants from the manuscript

I also wondered whether "endogenous Rab29" actually loses GDI binding and is poorly prenylated unlike other Rabs because the authors only used overexpressed, recombinant Rab29 proteins throughout this study.

Because Rab29 is expressed at low levels it would be very difficult to do this experiment with endogenous Rab29 using this assay. No other exogenously expressed Rabs show this phenotype.

3. Since lovastatin inhibits prenylation of all Rabs, it is important to test whether the observed effect on LRRK2 activation (Figure 2A) is directly related to inability to prenylate Rab29. The authors should test the effect of lovastatin on LRRK2 activation in the absence of Myc-Rab29 as a negative control.
This control is added in figure 2A, B
4. The authors prepared and used Rab29 D63A as a cytosolic-localized mutant, but it is not clear whether this mutant still functions as a Rab29 effector, especially when it is anchored to mitochondria (Figure 3). Co-IP experiments should be performed to test the interaction between LRRK2 and Rab29 WT or D63A.
Our experiments show that Rab29 D63A cannot bind LRRK2 in cells under conditions where wild type can (Fig. 7A,B).

5. Co-localization of LRRK2 and Rab10 in mito-Rab29-expressing cells is not clear at all (Figure 5A). The authors should stain the cells with appropriate organelle markers (Golgi and mitochondria) and quantify co-localization rates between Rab10 (or LRRK2) and each marker.

We have provided a new Fig. 3 with much better images and detailed co-localization quantitation.

Also, the phosphorylation rate of Rab10 should be evaluated by using the Phos-tag-based immunoblotting (see the authors' previous papers; eLife 2017;6:e31012 and Biochem. J. 2016;473:2671-2685) (Figure 5B).

This manuscript presents a huge amount of data and while characterization of precise phosphorylation rates would be ideal, this is not essential to the present story.

6. It is not clear how the authors quantify the percentage of cells with LRRK2 on mitochondria in Figure 6A. The authors need to describe the detailed method and add negative control data (i.e., without mito-Rab29). Also, typical images of cells in each sample should be provided in Figure 6B.
We have provided additional quantitation and Pearson's correlation coefficients and better colocalization as well (see new Fig. 7B and 6A).

(Other minor points)
7. MLi-2 treatment alone seemed to disrupt mitochondrial morphology and distribution in Figure 5. Is it true?

We apologize for any confusion and show better examples; MLi does not influence mito morphology.

8. For most of the immunoblot data, it is not clear which antibodies were used to detect immunoreactive bands. For example, was Myc-Rab29 detected with anti-Myc antibody or anti-Rab29 antibody in Figure 2A? The authors should include more detailed information in each figure legend.

The figure legends have been clarified and we apologize for this oversight.

Also, addition of lane numbers to immunoblot data is helpful for general readers.

We have added lane numbers to Fig. 9A as requested to make it easier for the reader. Space constraints make them harder to add elsewhere.

We thank the reviewers for their constructive comments that we believe have improved our story significantly.

August 7, 2019

Re: JCB manuscript #201902184R

Dr. Suzanne R. Pfeffer
Stanford University School of Medicine
Department of Biochemistry
279 Campus Drive
Stanford, CA 94305-5307

Dear Suzanne,

Thank you for submitting your revised manuscript entitled "Cytoplasmic diffusion between membrane compartments facilitates LRRK2 phosphorylation of Rab GTPases". The manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

You will see that reviewer #1 now supports publication, pending some relatively minor corrections (see her/his report below). However, reviewer #3 feels that, although the revised manuscript is improved, you have still not provided sufficient evidence that that LRRK2 moves between membrane compartments and suggests that you test this using the previously described cytosolic extraction method. S/he also feels that you restructure the paper entirely to focus on what s/he thinks are the more novel aspects of the study, namely: "artificially membrane-targeted LRRK2 can be activated without Rab29 and restricts the substrate Rab localization on the same membrane". While we appreciate this reviewer's points and agree that these issues need your response, we do not feel that further experiments will be necessary. In addition, we do not agree with the reviewer that the paper should be entirely refocused. Instead, we ask that you attempt to clarify in the text of the paper the conclusions that can be fully supported by the current dataset while illustrating the caveats of the less well-supported interpretations in the paper.

Our general policy is that papers are considered through only one revision cycle; however, given the enthusiasm of reviewers #1 and #2 (who had recommended acceptance after the first round) and the fact that the suggested changes are relatively minor, we are open to one additional short round of revision. Please note that we will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining comments of reviewers #1 and #3.

In the interests of speeding up subsequent processing of the paper, please also attend to the following formatting and style issues.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the

acceptance of your manuscript.**

1) Text limits: Character count for Articles and Tools 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.

2) Figures limits: Articles and Tools may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

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 also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

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 (at least in brief) in the text for readers who may not have access to referenced manuscripts.

6) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

7) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
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.).

8 References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

9) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

10) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

11) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, http://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

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

Sincerely,

Harald Stenmark, PhD
Monitoring Editor
JCB

Tim Spencer, PhD
Deputy Editor
Journal of Cell Biology

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

The revised manuscript is much improved, and I consider it acceptable for publication with minor changes as outlined below:

1. please eliminate various typos throughout the manuscript.

2. page 3, reference Madero-Perez et al, Mol. Neurodegener., 2018, correctly cites a publication related to the relocalization of phosphorylated Rabs to the mother centriole.
page 5, reference Madero-Perez et al, 2018, relates to the finding that a small pool of T21N Rab29 is localized to the Golgi (Madero-Perez et al, Front. Mol. Neurosci., 2018). Please add the latter reference and cite accordingly.

3. Figures 8E and 9B: the plasma membrane localization is still not convincing in both cases. Please additionally show an individual z-stack of the images where the PM localization is more obvious than in the maximal intensity projections currently depicted.

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

In the revised manuscript, the authors have improved the data quality by adding adequate control data and by performing quantification of the immunofluorescence images to assess co-localization. However, I feel that the major issue about the direct evidence for the diffusion model is not satisfactorily addressed.
The authors have now changed the title and clarified that LRRK2 moves by "cytoplasmic diffusion" rather than "membrane diffusion". However, if LRRK2 moves by cytosolic diffusion, I think that it is never surprising mechanism at all (as the reviewer #1 also pointed out in the comment #10). Many cytosolic kinases obviously work in this fashion (moving throughout the cytosol to phosphorylate distant substrates). Although a unique hypothesis for LRRK2 may be that once activated on the membrane by Rab29, LRRK2 detaches from that membrane and that this switching rely on Rab29 phosphorylation; however, such a mechanism has already been proposed and discussed in the previous report (by using a Rab29[T71E/S72E] mutant, EMBO J. 37:1-18, 2018). Unfortunately, no further experimental evidence is provided in the current paper. Furthermore, the expression "diffusion between membrane compartments" in the title would mislead readers into thinking that LRRK2 actively moves from organelles to organelles. If the authors want to claim that this mechanism actually occurs, I again request the direct evidence of LRRK2 translocation between membrane compartments (my original comment #1). I agree that the cytosolic portion of LRRK2 hinders visualization of membrane-localized LRRK2, but this may be solved by the previously used cytosolic extraction method ("freeze-thaw protocol" descried in EMBO J. 37:1-18, 2018). Otherwise, I would suggest the authors to modify the title and the abstract for more emphasizing other surprising and attractive data in this paper (e.g., artificially membrane-targeted LRRK2 became active [an increased pS1292 level] without Rab29 and restricted the substrate Rab localization on the same membrane) rather than the ambiguous "diffusion model".

August 30, 2019

RE: JCB Manuscript #201902184RR

Dr. Suzanne R. Pfeffer
Stanford University School of Medicine
Department of Biochemistry
279 Campus Drive
Stanford, CA 94305-5307

Dear Dr. Pfeffer:

Thank you for submitting your revised manuscript entitled "Cell biology of Rab GTPase phosphorylation by pathogenic LRRK2 kinase". 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.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles and Tools 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. You are below this limit at the moment but please bear it in mind when revising.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

3) 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 also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) Title: The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership. While we realize that you changed your title to accommodate the reviewer requests, we feel that the current title is a bit too vague really doesn't reflect the main message of the paper for a broader cell biology audience. Therefore we suggest the following title: "Membrane association but not identity is required for LRRK2 activation and phosphorylation of Rab GTPases". This is slightly over the 100 character limit, but we should be able to make an exception in this case.

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 (at least in brief) in the text for readers who may not have access to referenced manuscripts.

6) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

7) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
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.).

8) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

9) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures.
Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

10) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

11) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Harald Stenmark, PhD
Monitoring Editor
JCB

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