

Biochemical and structural characterization of Rab3GAP reveals insights into Rab18 nucleotide exchange activity

Corresponding Author: Dr Calvin Yip

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript by Farlie, Nguyen and colleagues reports the structural and biochemical investigation of the dimeric Rab3GAP complex, which has a dual function as GAP for Rab3 and GEF for Rab18. In addition to a negative stain characterization of the flexible intact complex, a cryo-EM reconstruction of a core complex that has full GEF activity towards Rab18 is presented. The interaction interface between the subunits RabGAP1 and RabGAP2 is verified by HDX. The experimental structure is combined with AlphaFold2 modeling, and in vitro GEF assays are employed to investigate Rab3GAP activity.

The main mechanistic conclusions of the paper are that Rab3GAP is auto-inhibited, which can be released by membrane binding, and that it forms a non-canonical interaction mode with its substrate Rab18, but these claims are poorly supported.

1. Several aspects about the analysis of the GEF assays should be addressed.

- The curves are normalized to a range from 1 to 0. As the extinction coefficient of mantGDP is roughly twice as large when bound to protein compared to in solution, the measurements should range from 1 (normalized at the zero time point) to ~0.5. It is therefore surprising that some curves e.g. in Fig 3d plateau at 0, some at 0.5.
- The triplicate measurements are averaged and presented as one curve. Ideally, each curve would be fit individually and the determined $k(\text{obs})$ can be given as average with standard deviation. This would allow a better comparison of experiments. Also, this is probably how the error bars on the data points in Figs 1d and 3b were determined?
- Why are the measurements from Figs 1c and 2c/3a not included in Fig 1d and 3b, respectively?
- The data in Fig 3a essentially reproduces the results of Figs 1c and 2c. Why does the data for WT Rab3GAP look different? And why does Rab3GAP look different yet again in Fig 3d?
- Without determination of $k(\text{obs})$, which depends on the slope and not the amplitude of the curves, or better $K(M)/k(\text{cat})$ for both conditions, the stimulation of GEF activity by presence of membrane is not convincingly demonstrated. The difference might actually be rather subtle.
- Similarly, the activity of the Rab3GAP core is only by a factor of 2.5 higher than the activity of the fl complex (Fig 3b), which may also result from a higher fraction of properly folded, active protein in the core preparation. An auto-inhibitory mechanism is possible, but is not compellingly demonstrated.
- Identical data appear in multiple figures (Fig 1d and 3b, 2c and 3a). Those data should be combined. Fig 2 can easily be integrated into Fig 1 and 3.

2. The model of Rab18 binding to Rab3GAP by AlphaFold2 is not convincing.

- Since the authors used AF2 models to interpret the EM density, it is not surprising that this model aligns well with the AF2 prediction of the dimeric complex. Importantly, the five top-ranked models AF2 for the trimeric complex show different binding modes of Rab18 relative to Rab3GAP and the PAE plots indicate very low confidence in the relative positioning. The main conclusion from Fig S6 should be that AF2 cannot reliably model the interaction of Rab3GAP and Rab18.
- The authors generated Rab18 mutants that could not be activated by Rab3GAP as efficiently as wild-type. Additional support for the proposed binding mode comes from the mapping of disease-causing mutations to this interface. This only shows that these residues may be involved in Rab3GAP-Rab18 interaction, but does not confirm the binding mode. Are these residues conserved in Rab18 and Rab3GAP1/2 homologs from different species?
- The findings do not exclude that Rab3GAP also binds (and remodels) the switch regions.
- Interactions of GEFs outside of the switch regions are not unique to Rab3GAP (see studies on the TRAPP complexes).

3. Additional point: In the introduction (l. 67-70), a nucleotide-selectivity of GEFs is suggested, which is not the case. Along these lines, why should Rab3GAP distinguish between the GTP- and GDP-loaded state of Rab18 (l. 382-383)?

Reviewer #2

(Remarks to the Author)

Rab3GAP1 and Rab3GAP2 were originally identified as subunits of a Rab3GAP complex that stimulates GTP hydrolysis by Rab3, but subsequent analysis has indicated that the Rab3GAP complex serves as a GEF for Rab18. Mutations in Rab3GAP cause the multisystem disorder Warburg micro syndrome. Fairlie et al. reported the GEF assay of recombinant Rab3GAP, overall structure of Rab3GAP by negative stain EM, structure determination of coreRab3GAP by cryoEM, HDX-MS analysis and works of the disease related mutations. Due to the failure of reconstitution of Rab3GAP-Rab18 complex, the predicted model by AlphaFold-multimer was employed, and the validity of this model was confirmed by mutation analysis.

Small GTPases are central intracellular regulators that exist in inactive and active states. The activity state is defined by binding to GDP or GTP, respectively. In order to activate these G-proteins, GEFs stimulate GDP release and the replacement of the GDP by GTP. Understanding the molecular events in the activation by GEFs is therefore a long-standing research topic.

Overall, the paper is solid and convincing, but the following concerns should be addressed.

1) GAP mechanism. The weak point of this paper is that authors explained the GAP mechanism using AlphaFold-multimer. Mutation analysis and sequence of alignment of Rab proteins might support this model. It is surprising that GEF functions not involving switch regions. Authors should discuss a more detailed GEF mechanism not involving switch regions (GDP release, GTP/GDP exchange, releasing mechanism of GTP-bound Rab18).

2) Autoinhibition mechanism by C-terminal region of Rab3GAP2 is interesting, and this autoinhibition could be released by membrane. Does the C-terminal region contain the membrane-associated domain/region, or exhibit characteristic features for the association of the membrane?

According to negative stain EM, C-terminal region is flexible. Is the closed form predominantly observed?

Does Alphafold predict the closed form (autoinhibited form)?

3) Please comment on GAP activity for Rab3 from the structural viewpoint.

Reviewer #3

(Remarks to the Author)

The authors present original work to elucidate mechanistic insights into the GEF activity of Rab3GAP against Rab18 and how disease-associated mutations of Rab3GAP affects its function. The studies include thorough and well-designed biochemical (enzymatic activity assay), mutagenesis and structural perspectives (cryoEM, HDX-MS, alphafold) to study the structure function relationship. Noteworthy results include:

- A high resolution cryoEM structure of Rab3GAP
- Functional data suggesting that full-length Rab3GAP complex is autoinhibited.
- The GEF activity of Rab3GAP is enhanced when its substrate, Rab18 is presented on a membrane or when Rab3GAP is devoid of its C-terminal domain.
- The C-terminal domain of the Rab3GAP2 subunit is not required for GEF activity and the Rab3GAP1 subunit alone cannot promote nucleotide exchange.
- The authors also propose a novel, non-canonical mechanism of interaction of Rab3GAP with Rab18 which do not involve the switch and interswitch regions based on alphafold predictions and mutagenesis.
- The three WMS-associated missense mutations disrupt Rab3GAP function potentially by altering the Rab18 binding site.

Overall, the study is well designed, thorough, relevant and very well written. The methodology is sound and meets expected standards in the field. It presents novel and impactful information and is worthy of publication if the following issues are addressed.

The authors show that the presence of C-terminal domain Rab3GAP2 reduces the catalytic efficiency from $6.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ to $2.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. However, it is not clear how biochemically or physiologically significant such a 3-fold change is. In the discussion, the authors discuss a mechanism for fine-tuning GEF activity by autoinhibition, taking the example of Rab5 GEF Rabex-5's autoinhibition and activation by Rabaptin. If the authors believe a 3-fold decrease constitutes autoinhibition as part of a fine-tuning mechanism, the authors should discuss further the amplitude of inhibition/activation in the context of that of Rabex-5. If quantitative data for Rabex-5 is not available, the authors should provide other arguments or temper the language regarding autoinhibition by the C-terminal of Rab3GAP2.

Minor issues:

Specific questions/comments:

Line 155: Supplemental Fig. 2d should be Supplemental Fig. 2c as described

Line 246: any specific reason why the deuteration experiments were performed at 18°C and not between 20 to 25°C?

Lines 256 – 258: In figure 5b, the color-coded texts say the exact same thing. I believe the red-coded text should be “significant difference...” instead of “no significant difference...”

In figure 5a, the text above the color-coded scale should be better worded for clarity. E.g. “decrease in Rab3GAP1 deuterium uptake when bound to Rab3GAP2”

Line 474: “In vitro GEF assay”, please provide reference for the previously described His-tagged Rabs purification or clarify where the previously described purification is.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have carefully addressed the concerns raised in the initial round of review. The data, their presentation and the discussion of the results are much improved, and so is the , and the manuscript overall.

Some minor points remain to be addressed prior publication:

- The structures in Supp Fig 6 are too small to compare the different models. A close-up of the Rab18-RabGAP3 interface of the superposition of five models would be helpful.
- For the localization studies of GFP RabGAP3 in Fig 6d an ER co-staining would be needed for a definite conclusion (or the data could be removed)-
- The data in Supp Fig 9c and 9d is very interesting and would be worth moving to the main figures.
- It should be pointed out in the legends if data is used in more than one figure.

Additional comments:

- A reference is incorrectly linked in line 398.

Reviewer #2

(Remarks to the Author)

The authors have addressed my previous concerns. I recommend the publication of this manuscript in Nature communications.

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We thank the editor and the four reviewers for taking the time to review our manuscript and providing critical feedback. We have made changes to the manuscript in response to these comments. Please find our responses below.

Reviewer #1:

“This manuscript by Farlie, Nguyen and colleagues reports the structural and biochemical investigation of the dimeric Rab3GAP complex, which has a dual function as GAP for Rab3 and GEF for Rab18. In addition to a negative stain characterization of the flexible intact complex, a cryo-EM reconstruction of a core complex that has full GEF activity towards Rab18 is presented. The interaction interface between the subunits RabGAP1 and RabGAP2 is verified by HDX. The experimental structure is combined with AlphaFold2 modeling, and in vitro GEF assays are employed to investigate Rab3GAP activity. The main mechanistic conclusions of the paper are that Rab3GAP is auto-inhibited, which can be released by membrane binding, and that it forms a non-canonical interaction mode with its substrate Rab18, but these claims are poorly supported.”

We are grateful for the detailed review of our manuscript from this reviewer.

Comment 1a: *Several aspects about the analysis of the GEF assays should be addressed.*

- The curves are normalized to a range from 1 to 0. As the extinction coefficient of mantGDP is roughly twice as large when bound to protein compared to in solution, the measurements should range from 1 (normalized at the zero time point) to ~0.5. It is therefore surprising that some curves e.g. in Fig 3d plateau at 0, some at 0.5.

Response: We apologize for the confusion. The curves obtained in all our GEF assays did plateau at around 0.5-0.6. The reason why the figures display curves plateauing around 0 is due to using the plateau value for background subtraction. We have reanalyzed all GEF assay data without performing background subtraction. The figures in the revised manuscript all have plateaus in the range of 0.5-0.6.

Comment 1b: *The triplicate measurements are averaged and presented as one curve. Ideally, each curve would be fit individually and the determined k_{obs} can be given as average with standard deviation. This would allow a better comparison of experiments. Also, this is probably how the error bars on the data points in Figs 1d and 3b were determined?*

Response: As recommended by this reviewer, we fit each curve individually and presented the k_{obs} for all experiments that involve comparing nucleotide exchange rates in the revised manuscript. To answer the reviewer's question, we determined the error bars in Figs 1d and 3b in the original manuscript using this approach.

Comment 1c:- *Why are the measurements from Figs 1c and 2c/3a not include in Fig 1d and 3b, respectively?*

Response: Fig 1c and 3a had typos in the figure legend. The concentration used was actually 444 nM, not the stated 400 nM. The reason why we did not include the measurement from 2c in Fig 3b was because the obtained data did not follow a linear trend. In particular, we noticed that k_{obs} began to plateau at higher concentrations of core Rab3GAP. As a result, the data generated could

not be used for determining catalytic efficiency. We have now repeated these experiments but used GEF at concentration from 0 nM to 300 nM instead. Using this lower range of GEF enabled us to obtain data that follow a linear trend.

Comment 1d:- *The data in Fig 3a essentially reproduces the results of Figs 1c and 2c. Why does the data for WT Rab3GAP look different? And why does Rab3GAP look different yet again in Fig 3d?*

Response: We believe that the discrepancy was caused by the way we performed background subtraction. More specifically, we took the value of the plateau for background subtraction because this should represent the point where no Mant-GDP is bound. However, upon further inspection, we noticed that some assays never plateaued at the concentrations used in our experiments. To resolve this problem, we repeated the analysis of all GEF assay data without performing the background subtraction step.

Comment 1e:- *Without determination of $k(\text{obs})$, which depends on the slope and not the amplitude of the curves, or better $K(M)/k(\text{cat})$ for both conditions, the stimulation of GEF activity by presence of membrane is not convincingly demonstrated. The difference might actually be rather subtle.*

Response: As suggested by the reviewer, we performed dose-response curves of FL and core Rab3GAP in the presence of membranes to determine the catalytic efficiency. We found that membranes significantly enhanced GEF activity for both FL and core Rab3GAP. We appreciate this suggestion as it allowed us to perform a more diligent analysis of the effect of membranes on Rab3GAP activity.

Comment 1f:- *Similarly, the activity of the Rab3GAP core is only by a factor of 2.5 higher than the activity of the fl complex (Fig 3b), which may also result from a higher fraction of properly folded, active protein in the core preparation. An auto-inhibitory mechanism is possible, but is not compellingly demonstrated.*

Response: Although the higher level of GEF activity could be due to a higher level of active complex in our prep, we feel that the likelihood is low. In particular, our negative stain analysis of FL Rab3GAP and core Rab3GAP showed that both are well-behaved in solution with no apparent aggregation or misfolding. That said, we agree that the data we presented suggested that auto-inhibition is possible but more research would be needed to delineate this potential regulatory mechanism of Rab3GAP. We have revised the text and the title of the manuscript to more appropriately capture this point.

- Identical data appear in multiple figures (Fig 1d and 3b, 2c and 3a). Those data should be combined. Fig 2 can easily be integrated into Fig 1 and 3.

Response: The data from Fig 2 could be integrated into Figures 1 and 3, but we felt that the flow of the manuscript could be enhanced if these data were presented in separate sections.

Comment 2a:

- Since the authors used AF2 models to interpret the EM density, it is not surprising that this model aligns well with the AF2 prediction of the dimeric complex.

Response: To clarify, we fit the AlphaFold2-predicted models of Rab3GAP1 and Rab3GAP2 one by one into the experimental cryo-EM density map and then use this as a starting point to construct the structural model of core Rab3GAP. We carried out a separate AlphaFold-Multimer run afterwards to test the ability of the AlphaFold-Multimer algorithm to predict the structure of core Rab3GAP. The observation that the predicted model aligns well with the experimentally determined structure of core Rab3GAP reassured us about the suitability of applying AlphaFold-Multimer to model Rab18 binding.

Comment 2b: *Importantly, the five top-ranked models AF2 for the trimeric complex show different binding modes of Rab18 relative to Rab3GAP and the PAE plots indicate very low confidence in the relative positioning. The main conclusion from Fig S6 should be that AF2 cannot reliably model the interaction of Rab3GAP and Rab18.*

Response: To address the reviewer's concern, we performed a new modelling run of core Rab3GAP in complex Rab18 using the recently released AlphaFold3. All 5 models generated from AlphaFold3 resemble one another and show Rab3GAP binding Rab18 at an atypical interface away from the switch regions. Each of every one of these models has ipTM score of 0.8, indicating a high-quality prediction for protein-protein interfaces. Additionally, we displayed the models using the colour by PAE domains feature in ChimeraX, which groups regions of low PAE (<5 Ångstroms) by colour. The observation that Rab18 and the Rab3GAP1+2 interface were grouped within the same PAE domain provides further confidence in the predicted model of the complex.

Comment 2c: *The authors generated Rab18 mutants that could not be activated by Rab3GAP as efficiently as wild-type. Additional support for the proposed binding mode comes from the mapping of disease-causing mutations to this interface. This only shows that these residues may be involved in Rab3GAP-Rab18 interaction, but does not confirm the binding mode. Are these residues conserved in Rab18 and Rab3GAP1/2 homologs from different species? The findings do not exclude that Rab3GAP also binds (and remodels) the switch regions.*

Response: To answer the reviewer's question, we have conducted multiple sequence alignment analyses and showed that the Rab3GAP and Rab18 residues predicted to be involved in intermolecular interaction are conserved. We presented a section of the Rab alignment in the original manuscript (Fig 6e in the original manuscript). We agree with the reviewer that our data could not definitively prove that the residues observed in the original AlphaFold-Multimer and the new AlphaFold3-predicted models are indeed involved in binding. However, we were unable to carry out HDX-MS to validate the predicted interface due to the inherent low affinity between Rab18 and Rab3GAP. To address this issue, we generated a new Rab3GAP variant containing Rab3GAP1 subunit devoid of its GAP domain to explore the possibility that other parts of the complex are involved in binding the switch regions of Rab18. Our results showed that the GAP domain is only required for the Rab3a GAP activity of Rab3GAP but not its Rab18 GEF activity. To provide additional supportive data, we carried out confocal microscopy experiments and

demonstrated that the ER localization of Rab18 in cells is disrupted by the same mutation we have shown to block its activation by Rab3GAP. Lastly, we revised the discussion section to describe the possible involvement of other parts of Rab3GAP in mediating interaction with Rab18.

Comment 2e: *Interactions of GEFs outside of the switch regions are not unique to Rab3GAP (see studies on the TRAPP complexes).*

Response: Interactions outside of the switch regions have indeed been observed for other GEFs but these GEFs also contact the nucleotide-binding domain of their cognate Rabs. What is unusual about Rab3GAP is that based on the AlphaFold-Multimer/AlphaFold3 predicted models, there does not appear to be a region of this complex that is located at a sufficiently close distance to contact the switch regions.

Comment 3: *Additional point: In the introduction (l. 67-70), a nucleotide-selectivity of GEFs is suggested, which is not the case. Along these lines, why should Rab3GAP distinguish between the GTP- and GDP-loaded state of Rab18 (l. 382-383)?*

Response: We apologize for these inaccurate statements. We have corrected these in the revised introduction.

Reviewer #2:

“Rab3GAP1 and Rab3GAP2 were originally identified as subunits of a Rab3GAP complex that stimulates GTP hydrolysis by Rab3, but subsequent analysis has indicated that the Rab3GAP complex serves as a GEF for Rab18. Mutations in Rab3GAP cause the multisystem disorder Warburg micro syndrome. Fairlie et al. reported the GEF assay of recombinant Rab3GAP, overall structure of Rab3GAP by negative stain EM, structure determination of coreRab3GAP by cryoEM, HDX-MS analysis and works of the disease related mutations. Due to the failure of reconstitution of Rab3GAP-Rab18 complex, the predicted model by AlphaFold-multimer was employed, and the validity of this model was confirmed by mutation analysis. Small GTPases are central intracellular regulators that exist in inactive and active states. The activity state is defined by binding to GDP or GTP, respectively. In order to activate these G-proteins, GEFs stimulate GDP release and the replacement of the GDP by GTP. Understanding the molecular events in the activation by GEFs is therefore a long-standing research topic. Overall, the paper is solid and convincing, but the following concerns should be addressed.”

We are grateful for the positive feedback from this reviewer.

Comment 1: GAP mechanism. The weak point of this paper is that authors explained the GAP mechanism using AlphaFold-multimer. Mutation analysis and sequence of alignment of Rab proteins might support this model. It is surprising that GEF functions not involving switch regions. Authors should discuss a more detailed GEF mechanism not involving switch regions (GDP release, GTP/GDP exchange, releasing mechanism of GTP-bound Rab18).

Response: We believe the reviewer was referring to the GEF mechanism in this comment. We have conducted a multiple sequence alignment of Rab proteins (results shown in Figure 6e of the

original manuscript). This analysis showed that the Rab18 residues predicted to interact with Rab3GAP are not conserved in other Rab proteins. In the revised manuscript, we added a supplementary figure that displayed the full alignment of Rab18 with other Rabs and one that showed the alignment of Rab3GAP subunits from different species. Furthermore, to determine if other regions of Rab3GAP might be involved in engaging Rab18, we generated a variant of core Rab3GAP lacking the putative GAP domain in the Rab3GAP1 subunit. This mutation did not disrupt Rab18 GEF activity. In the revised discussion section, we commented on the implication of this new finding. We also cited a recent report that shows that mutating allosteric sites away from the switch regions of Gsp1/Ran could cause this small GTPase to change its nucleotide preference. Interestingly, these “allosteric residues” of Gsp1/Ran mapped to locations near the predicted Rab3GAP binding regions to Rab18. In summary, while our studies did not arrive at a definite GEF mechanism of Rab3GAP, results from our *in silico* modelling, multiple sequence alignment, and site-specific mutagenesis pointed to the possibility of Rab3GAP inducing Rab18 nucleotide exchange through an allosteric mechanism.

Comment 2: *Autoinhibition mechanism by C-terminal region of Rab3GAP2 is interesting, and this autoinhibition could be released by membrane. Does the C-terminal region contain the membrane-associated domain/region, or exhibit characteristic features for the association of the membrane?*

Response: We have conducted a more comprehensive kinetic analysis and we found that core Rab3GAP also shows enhanced GEF activity in the presence of membranes as well. This finding necessitated us to revise our original proposal that the presence of a membrane releases Rab3GAP from autoinhibition. To answer the reviewer’s question, there were no obvious membrane binding domains present in the C-terminal domain of Rab3GAP2 but the AlphaFold2-predicted structural model of the Rab3GAP2 subunits shows the presence of a cluster of basic residues at its C-terminal domain of Rab3GAP2 (refer to supplementary Figure 11). However further work will be required to determine if these residues indeed function to mediate potential membrane association.

Comment 3: *According to negative stain EM, C-terminal region is flexible. Is the closed form predominantly observed?*

Response: Based on our negative stain EM 2D analysis, fewer than 10% of particles belong to the fully closed conformation shown in the figure. However, it is conceivable that a number of the open conformations would also occlude the Rab18 substrate binding region and block GEF activity. Lastly, we cannot rule out that some of the open conformations resulted from the flattening effect of the negative stain EM specimen preparation procedure.

Comment 4: *Does Alphafold predict the closed form (autoinhibited form)?*

Response: The two domains of the Rab3GAP2 subunit are connected by a flexible linker. As a result, AlphaFold2 and AlphaFold3 have low confidence in predicting the precise orientation of the two domains. This is illustrated by the high PAE scores for residues in the C-terminal domain of Rab3GAP2, indicating low confidence in the position of this domain relative to the rest of the complex.

Comment 5: Please comment on GAP activity for Rab3 from the structural viewpoint.

Response: The GAP domain of Rab3GAP is located in the flexible C-terminal region of the Rab3GAP1 subunit. When we were investigating the potential involvement of other regions of Rab3GAP in Rab18 binding and nucleotide exchange, we examined a Rab3GAP mutant devoid of this GAP domain. We found that this mutant retains Rab18 GEF activity but lost its GAP activity towards Rab3a. This finding suggests that the Rab3a GAP activity and Rab18 GEF activity are located in distinct sites of the complex. We also carried out AlphaFold-Multimer and AlphaFold3 analysis, both of which showed that the Rab3GAP1 subunit binds the switch regions of Rab3a which is consistent with results from a previous study showing that Switch I of Rab3a engages the GAP domain of Rab3GAP and that R728 of Rab3GAP1 and Q81 of Rab3a provide catalytic residues in a mechanism similar to Ras and Rho GAPs (Clabecq *et al.*, 2000).

Reviewer #3:

The authors present original work to elucidate mechanistic insights into the GEF activity of Rab3GAP against Rab18 and how disease-associated mutations of Rab3GAP affects its function. The studies include thorough and well-designed biochemical (enzymatic activity assay), mutagenesis and structural perspectives (cryoEM, HDX-MS, alphafold) to study the structure function relationship. Noteworthy results include:

- A high resolution cryoEM structure of Rab3GAP*
- Functional data suggesting that full-length Rab3GAP complex is autoinhibited.*
- The GEF activity of Rab3GAP is enhanced when its substrate, Rab18 is presented on a membrane or when Rab3GAP is devoid of its C-terminal domain.*
- The C-terminal domain of the Rab3GAP2 subunit is not required for GEF activity and the Rab3GAP1 subunit alone cannot promote nucleotide exchange.*
- The authors also propose a novel, non-canonical mechanism of interaction of Rab3GAP with Rab18 which do not involve the switch and interswitch regions based on alphafold predictions and mutagenesis.*
- The three WMS-associated missense mutations disrupt Rab3GAP function potentially by altering the Rab18 binding site.*

Overall, the study is well designed, thorough, relevant and very well written. The methodology is sound and meets expected standards in the field. It presents novel and impactful information and is worthy of publication if the following issues are addressed.

We are grateful for the positive feedback from this reviewer.

Comment 1: *The authors show that the presence of C-terminal domain Rab3GAP2 reduces the catalytic efficiency from $6.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ to $2.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. However, it is not clear how biochemically or physiologically significant such a 3-fold change is. In the discussion, the authors discuss a mechanism for fine-tuning GEF activity by autoinhibition, taking the example of Rab5 GEF Rabex-5's autoinhibition and activation by Rabaptin. If the authors believe a 3-fold decrease constitutes autoinhibition as part of a fine-tuning mechanism, the authors should discuss further the amplitude of inhibition/activation in the context of that of Rabex-5. If*

quantitative data for Rabex-5 is not available, the authors should provide other arguments or tamper the language regarding autoinhibition by the C-terminal of Rab3GAP2.

Response: Previous studies showed that activation of the Rab5 GEF Rabex-5 by Rabaptin is approximately 2- to 3-fold. Additionally, truncation mutations engineered to create a catalytic core Rabex-5 also increase activity by a similar level as Rabaptin. The magnitude of the increase in catalytic activity is in line with what we observed for core Rab3GAP and full-length Rab3GAP. We have added more details in the Rabex-5 comparison in the revised discussion section. As recommended by this reviewer, we have adjusted the language on autoinhibition by the C-terminal domain of Rab3GAP2 as we do not fully understand the precise regulatory mechanism.

Comment 2a: *Line 155: Supplemental Fig. 2d should be Supplemental Fig. 2c as described*

Response: We apologize for this mistake. We have corrected this accordingly.

Comment 2b: *Line 246: any specific reason why the deuteration experiments were performed at 18°C and not between 20 to 25°C?*

Response: This was the ambient temperature in the room where the deuteration experiment was performed.

Comment 2c: *Lines 256 – 258: In figure 5b, the color-coded texts say the exact same thing. I believe the red-coded text should be “significant difference...” instead of “no significant difference...”*

Response: We apologized for this error. We have corrected the colour-coded text.

Comment 2d: *In figure 5a, the text above the color-coded scale should be better worded for clarity. E.g. “decrease in Rab3GAP1 deuterium uptake when bound to Rab3GAP2*

Response: We thanked the reviewer for this suggestion. We have changed the wording to “Decrease in Rab3GAP1 deuterium incorporation when bound to Rab3GAP2”.

Comment 23: *Line 474: “In vitro GEF assay”, please provide reference for the previously described His-tagged Rabs purification or clarify where the previously described purification is.*

Response: We apologize for this confusion. We have revised the text and it now stated that the His-tagged Rabs were purified as described in the methods section.

Reviewer #4:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We are grateful for the help provided by this reviewer.