

Structural basis for Rab6 activation by the Ric1-Rgp1 complex

Corresponding Author: Dr J. Fromme

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)

Feathers, Vignogna and Fromme report the structural and biochemical analysis of the Ric1-Rgp1 GEF complex bound to its substrate GTPase Rab6. Based on a cryo-EM structure determined by SPA, the authors define a novel RabGEF fold and shine light on its function on a molecular level. The main findings of the study comprise a) the identification of the nucleotide exchange mechanism, which relies on an interaction of the GEF with the Switch I region that enforces a severe conformational change with displacement of some residues of up to 25 Å and thus stabilizes the GTPase in a nucleotide free intermediate state, b) evidence for the importance of the HVD in substrate recognition and specificity. c) characterization of a novel domain (GEF Binding Element, GBE), of which the exact function remains to be identified.

The insight derived from the structure is validated thoroughly by in vitro and in vivo assays, putting together a compelling and clearly presented study, which warrants publication.

However, several points should be addressed and further discussed prior publication:

- Two of the major findings are the molecular interactions of the Rab6 HVD and the GBE, both of which are connected via flexible unresolved linker regions to the remainder of the structure. Because sequence assignment might be challenging at the given resolution, depicting the map for these regions would be helpful to assess the reliability of the build model and should be included in already existing figures (e.g. 4b for the HVD).

In addition, a depiction of resolved and unresolved parts of the EM structure may be beneficial (e.g. in Figure 1e)

- The importance of the HVD is further stressed by the experiments with HVD- chimeras. Strikingly, the interacting motif overlaps largely with the conserved CIM motif of Rabs that is also recognized by REP/GDI. The Rgp1-interacting motif comprises the CIM, +3 residues up and downstream, with the CIM being the best conserved stretch within that region. Because the CIM motif is conserved within other Rab GTPases as it serves as a recognition motif for lipidation, this raises the question why other GTPases, and the Rab6-HVD5 chimera in particular, would not be recognized by Ric1/Rgp1. This issue might be discussed along with a comparison of Rab6 and Rab5 HVD sequences. Is it possible that in addition to specific sequence motifs other factors like length and therefore distance to membrane or the G domain play a role. Additionally, the authors could discuss how (competitive) binding of this sequence motif by both GDI/REP and GEF might play a role in membrane association of the GTPase. Could Ric1-Rgp1 - and maybe other GEFs – have a GDF-like function?

- The authors may elaborate on their thoughts regarding the GBE domain. Do they assume it functions as a scaffold to stabilize the GEF domain? Does it add another layer of regulation by activating the GEF domain? Are there highly conserved residues that might suggest a protein:protein interaction interface?

- The authors identify a potential amphipathic helix in Rgp1 and speculate about its function in protein-membrane interaction. A helical wheel projection of the corresponding residues together with predicted physicochemical properties like hydrophobic moment might strengthen the hypothesis.

side note: in Figure S4c the construct is referred to as Δ84-95 whereas in every other figure (S4b, 5e, 5f) it is referred to ΔAH

- Some of the figures lack proper labelling and annotations. E.g. Figure S1 a, c, d, e should include molecular weight markers for SDS-PAGE-gels and a scale on the axis for the SEC chromatogram. Moreover, column type and buffer used in the experiment are not stated in the caption.

- There are some typos into the methods section: mostly missing spaces between value and unit (sections: “Prenylated Rab6-mantGDP-Gdi1 preparation” and “GEF activity assay”)

- The sentence “We refer to this C-terminal α-solenoid domain of Ric1 as the GEF domain (Fig. 1e) as we have biochemically established its nucleotide exchange activity as described further below.” in paragraph 3 of the Results section may be misleading. It suggests that there was a functional minimal GEF complex established comprising only that very GEF domain, even though the experiments apparently were done with the full-length protein.

Reviewer #2

(Remarks to the Author)

The manuscript presented by Feathers et al. reports the structure of a ternary complex consisting of the guanine nucleotide exchange factor Ric1-Rgp1 and its cognate GTPase-substrate Rab6 resolved by cryo-electron microscopy. Based on this structure the authors describe an unknown GEF domain and compare it to other, known GEF domains. They highlight residues in this specific domain important for GEF activity including substrate recognition and deduce a mechanism for GEF-mediated nucleotide exchange. Also, the authors observe an interaction between the GEF-complex and the unstructured C-terminal half of Rab6, the so-called hypervariable domain (HVD). This suggests an interesting additional layer of recognition between GEF and Rab-GTPase. The authors test the importance of this HVD recognition for Rab6 activation by Ric1-Rgp1 in vivo utilizing an HVD-swap mutant replacing the HVD of Rab6 by the Rab5-HVD. Furthermore, they propose a potential amphipathic helix in the subunit Rgp1. The authors describe this helix as important but not essential for correct cellular GEF localisation. Taken together the presented findings provide substantial new insights into the activation mechanism of the Rab6-GEF Ric1-Rgp1 and its cellular localization.

The study presented here is clearly written and the results are of high quality. The obtained data support the conclusions the authors make. The manuscript describes very interesting new insights deduced from the presented cryo-EM structure and tested in well-conceived biochemical and cellular experiments. These new insights will be of interest to a broad readership interested in Golgi organization, membrane trafficking and GEF-Rab interaction and activation.

Overall, the study of Feathers et al. is of very good quality, the rationale, the described results and the presentation of data are easy to follow and understand. However, some of the conclusions are based on a very thin layer of biochemical data and need to be addressed:

1) The GEF assay presented in Figure 2 needs some additional controls. The authors should test for the intrinsic nucleotide exchange rate of Rab6 under these conditions. Is there remaining activity of the GEF-domain mutant or is the observed rate identical to the intrinsic exchange rate of Rab6 without GEF? In this context also a titration of the wild type vs. the mutant GEF complex would be of interest. Is there residual activity of the mutant complex which would only be visible if higher amounts of the GEF were tested?

In the context of the GEF assay, the authors also might want to control the membrane association of the GEF complex. Is it a loss of GEF activity or is there a problem with the membrane association of the mutant GEF-complex in the in vitro assay? However, the in vivo data suggests that the mutant GEF-complex is binding to membranes, but as the authors point out this might also be due to the action of Rab33 as a recruiter which is missing in the in vitro GEF assay.

2) The in vivo experiments presented in Figure 4 suggest that “the chimeric GFP-Rab6-HVDRab5 was significantly mis-localized,...” (page 11, top paragraph) which was not leading to a growth defect at 38°C (Fig S3c). To narrow down the impact of the HVD-swap on Rab6 activation the authors should use their in vitro GEF-assay to measure activation rates of Ric1-Rgp1 towards wildtype and HVD-swap Rab6. This will allow the authors to get an idea of how much the HVD contributes to the GEF-Rab affinity.

3) Based on AlphaFold predictions the authors suggest the presence of an amphipathic helix. Deletion of the according amino acids leads to a loss of localization of Rgp1-GFP in vivo. However, they do not provide any evidence that this alpha helix has an amphipathic character. I would suggest presenting a helical wheel representation and calculating the hydrophobic moment of that helix. Additionally to the presented deletion, a mutational analysis of this helix should be included to test the hypothesis of an amphipathic helix and to verify the interpretation of the deletion mutant. Usually, the membrane association of proteins with potential amphipathic helices is also tested in membrane association assay, where the wildtype protein and mutant proteins are compared.

4) Optionally the authors could also assess if the membrane association mediated by the amphipathic helix is necessary for the observed GEF-activity in vitro using again wildtype and mutant proteins. Since Rab33 is not present in their GEF-assay this would address if there are other membrane association cues in the Rab6 GEF-complex or, like the authors suggest in their discussion, the complex only relies on the suggested amphipathic helix and the interaction to Rab33.

Reviewer #3

(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.

Reviewer #4

(Remarks to the Author)

The article by Feathers et al describes the molecular basis for how the Ric1-Rgp1 complex activates the Rab6 GTPase. This is a highly conserved complex through evolution and mechanistic information of how it mediates nucleotide exchange is a useful contribution. The authors identified a previously uncharacterised helical domain that mediates GEF exchange, as

well as an unexpected interaction of an arrestin fold of the GEF with the hypervariable domain of Rab6.

Overall this is clearly presented study that provides novel insight into an important GEF that regulates the Golgi complex. I have some suggestions that will be required to clarify and validate some of the analysis, and with this I am strongly supportive of publication in Nature Communications.

Major Revisions

1. Thank you to the authors for providing the map and pdb files for analysis. One of the things missing in the manuscript is the correlation between the map and the model, especially for the residues mediating the interface. I would strongly suggest showing a clear zoom in on the interface where the map is shown for these residues (essentially the zoom in shown in Fig 2C, except in this figures showing the map for these regions as a new supplemental figure). Essentially show the figure in S2D, but with a clear focus on the interfacial residues at the GEF domain.

2. In addition it would also be very useful to see the sequence alignment of the helices in Ric1 that mediate GEF activity across evolution, as this would clearly additionally highlight the important role of F860 and R912. I recommend adding this as a panel to Figure 2.

3. The authors claim that the F860A-R912A complex is catalytically dead. Do the authors know that the F860A-R912A complex is catalytically dead? Without the control experiment in panel 2D showing no GEF present, we can't determine if the GEF is dead (ie same activity as no GEF present). please rephrase or show the control experiment. Please include the experimental conditions for this assay in the figure legend of Fig 2D (concentrations [nucleotide, GEF, Rab, and membrane], and membrane composition) so readers don't need to chase this down in the methods.

4. Have the authors tried a DALI search to see the closest fold to the helical domain of Ric1? As it does not share homology to other GEFs this could be useful information to include.

5. The discovery of the arrestin fold in Rgp1 binding to the HVT is an intriguing discovery. It would be useful for the authors to mutate residues in the HVT and test their in vitro GEF assay. This would likely have to be done in the absence of prenylation, as it would also disrupt this. Does the Ric-RGP complex still have GEF activity in the absence of membrane? If so it would be worth trying GEF assays with and without the mutated HVT as a proof of this altering GEF activity. The cell experiments are nice, but may be working through a differential mechanism.

6. It would also be useful to show the sequence conservation of the interacting residues in the HVT in Fig4, instead of in the supplement as this is something that I think warrants being in the main figures.

7. It would be useful for the authors to redo their alphafold multimer search with recently released alphafold3.
Minor revision.

1. The GEF assay shown in Fig 2D is very hard to read with fonts being extremely small, please improve the clarity of this figure.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Thanks to the authors for carefully addressing all points raised in the original review and congratulations on this interesting study, which should be published without further delay.

Reviewer #2

(Remarks to the Author)

I am pleased to report that the authors have effectively addressed all my concerns and have incorporated previously missing experimental data into their manuscript. I recommend publishing this study, as it offers highly interesting new insights and is significant to the field.

Reviewer #3

(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.

Reviewer #4

(Remarks to the Author)

The authors have done an excellent job in repsonding to my queries, as well as those from the additional reviewers. I am strongly supportive of publication, and congratulate all authors on a very nice contribution to Rab-GEF biology.

Signed
John Burke

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We thank the reviewers for their support of our work as well as their suggestions for improvement. The revised manuscript now includes the results of new experiments, additional structural figures and figure panels, and text revisions in order to address the concerns of the reviewers. We think the result is a further improved manuscript that provides even stronger support to our conclusions while presenting a significant number of important findings:

- The first structure of the Ric1-Rgp1 complex
- The first structure of Ric1-Rgp1 caught in the act of Rab6 nucleotide exchange
- Characterization of a novel GEF domain
- The mechanism of Rab6 nucleotide exchange by Ric1-Rgp1
- Discovery that the Rab6 HVD binds to Rgp1
- Characterization of the importance of the Rab6 HVD for activation *in vitro* and localization *in vivo*
- Identification of a putative membrane-binding element in Rgp1 that is important for Golgi localization
- A model for Ric1-Rgp1 activation of Rab6 on the Golgi membrane

REVIEWER COMMENTS

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Feathers, Vignogna and Fromme report the structural and biochemical analysis of the Ric1-Rgp1 GEF complex bound to its substrate GTPase Rab6. Based on a cryo-EM structure determined by SPA, the authors define a novel RabGEF fold and shine light on its function on a molecular level. The main findings of the study comprise a) the identification of the nucleotide exchange mechanism, which relies on an interaction of the GEF with the Switch I region that enforces a severe conformational change with displacement of some residues of up to 25 Å and thus stabilizes the GTPase in a nucleotide free intermediate state, b) evidence for the importance of the HVD in substrate recognition and specificity. c) characterization of a novel domain (GEF Binding Element, GBE), of which the exact function remains to be identified. The insight derived from the structure is validated thoroughly by *in vitro* and *in vivo* assays, putting together a compelling and clearly presented study, which warrants publication.

We thank the reviewer for their strong support of our study.

However, several points should be addressed and further discussed prior publication:

- Two of the major findings are the molecular interactions of the Rab6 HVD and the GBE, both of which are connected via flexible unresolved linker regions to the remainder of the structure. Because sequence assignment might be challenging at the given resolution, depicting the map for these regions would be helpful to assess the reliability of the build model and should be included in already existing figures (e.g. 4b for the HVD). In addition, a depiction of resolved and unresolved parts of the EM structure may be beneficial (e.g. in Figure 1e)

We have now included a new figure (new Supplementary Fig. S3) to address this suggestion and a similar suggestion from Reviewer 4. It shows the cryoEM density of three additional regions: the Rab6 switch I region, the Rab6 HVD, and the Ric1 GBE.

The GBE region of the map has the lowest resolution as this is the most distal portion of the GEF domain. When re-inspecting our model and map, we decided that we should model this lower-resolution region as a poly-ALA model (Chain A, 998-1057; Chain B, 379-409). We have uploaded the revised PDB file to the RCSB, updated the statistics in Table 1, included the updated validation report with this revision, and made notes of this in the manuscript text. This has no impact on any of our interpretations or conclusions.

As suggested, we also now include a depiction of which portions of the complex are modeled or disordered in Fig. 1e.

- The importance of the HVD is further stressed by the experiments with HVD-chimeras. Strikingly, the interacting motif overlaps largely with the conserved CIM motif of Rabs that is also recognized by REP/GDI. The Rgp1-interacting motif comprises the CIM, +3 residues up and downstream, with the CIM being the best conserved stretch within that region. Because the CIM motif is conserved within other Rab GTPases as it serves as a recognition motif for

lipidation, this raises the question why other GTPases, and the Rab6-HVD5 chimera in particular, would not be recognized by Ric1/Rgp1. This issue might be discussed along with a comparison of Rab6 and Rab5 HVD sequences. Is it possible that in addition to specific sequence motifs other factors like length and therefore distance to membrane or the G domain play a role. Additionally, the authors could discuss how (competitive) binding of this sequence motif by both GDI/REP and GEF might play a role in membrane association of the GTPase. Could Ric1-Rgp1 - and maybe other GEFs – have a GDF-like function?

Although the CIM motif is indeed conserved among the different Rab GTPases, it appears there are slight differences on the edges of the motif that could potentially distinguish them. For example, in yeast the Ypt6 motif is ...VIDIS... and the Vps21 motif is ...RVDLN... . However, these differences are not precisely conserved across organisms in all kingdoms, perhaps due to some co-evolution of the HVD sequences with their GEFs. There are also slight differences in the lengths of the Rab6 vs Rab5 HVDs on the C-terminal side of their CIMs, but only a difference of ~2 amino acids, which we would guess is not significant enough of a difference to provide robust specificity.

Regarding the possibility that Ric1-Rgp1 can act as a GDF to displace Rab6 substrate from GDI, we believe the available structural data would argue against this possibility. The hydrophobic sidechains of the CIM motif interact directly with GDI and also with Rgp1. Therefore, the Rab6 HVD would need to dissociate from GDI before it can bind to Rgp1. To function as a GDF, the GEF would need to bind to the Rab-GDI complex and form a transient tripartite GEF-Rab-GDI complex (just as GEFs bind to Rab-GDP complexes and form a transient tripartite GEF-Rab-GDP) complex. We have examined multiple different ways of superimposing GEF-Rab and Rab-GDI complexes (i.e. superimposing different portions of the HVD or the NBD) and there is always a significant steric clash, so we have no evidence to support a GDF role based on the existing structural data. Of course it is possible that the GEF serves as a GDF through some other mechanism, perhaps by binding to another surface of GDI to destabilize Rab binding, but at this point we do not feel comfortable speculating one way or the other.

Therefore, we have added the following sentence to the Discussion to address the reviewers point about the conservation of the CIM motif: “*The*

three-residue CIM motif within the HVD is well conserved across Rab family members, but the sequences surrounding this motif are distinct.

- The authors may elaborate on their thoughts regarding the GBE domain. Do they assume it functions as a scaffold to stabilize the GEF domain? Does it add another layer of regulation by activating the GEF domain? Are there highly conserved residues that might suggest a protein:protein interaction interface?

We assume the GBE may simply stabilize the GEF domain fold. There is no obvious conserved region on the solvent-exposed surface of the GBE (although the buried surface of the GBE that interacts with the Ric1 GEF domain is well conserved).

- The authors identify a potential amphipathic helix in Rgp1 and speculate about its function in protein-membrane interaction. A helical wheel projection of the corresponding residues together with predicted physicochemical properties like hydrophobic moment might strengthen the hypothesis.

We have added a helical wheel projection to Figure 5.

side note: in Figure S4c the construct is referred to as $\Delta 84-95$ whereas in every other figure (S4b, 5e, 5f) it is referred to ΔAH

Thank you for noticing this labeling inconsistency. We have fixed Figure S4c to match the other figures.

- Some of the figures lack proper labelling and annotations. E.g. Figure S1 a, c, d, e should include molecular weight markers for SDS-PAGE-gels and a scale on the axis for the SEC chromatogram. Moreover, column type and buffer used in the experiment are not stated in the caption.

We have now added the requested information to Figure S1 and the corresponding legend.

- There are some typos into the methods section: mostly missing spaces between value and unit (sections: “Prenylated Rab6-mantGDP-Gdi1 preparation” and “GEF activity assay”)

Thank you for noticing this, we have fixed the typos.

- The sentence “We refer to this C-terminal α -solenoid domain of Ric1 as the GEF domain (Fig. 1e) as we have biochemically established its nucleotide exchange activity as described further below.” in paragraph 3 of the Results section may be misleading. It suggests that there was a functional minimal GEF complex established comprising only that very GEF domain, even though the experiments apparently were done with the full-length protein.

Thank you for pointing this out, it was not our intention to imply that we established the sufficiency of this domain for GEF activity. The mutational analysis we performed and presented in Figure 2F establishes that this domain, which we identified by cryoEM, possesses residues that are biochemically required for GEF activity. We have therefore reworded this sentence to read “... as we have identified residues within this domain required for nucleotide exchange activity as described further below.”

Reviewer #2 (Remarks to the Author):

The manuscript presented by Feathers et al. reports the structure of a ternary complex consisting of the guanine nucleotide exchange factor Ric1-Rgp1 and its cognate GTPase-substrate Rab6 resolved by cryo-electron microscopy. Based on this structure the authors describe an unknown GEF domain and compare it to other, known GEF domains. They highlight residues in this specific domain important for GEF activity including substrate recognition and deduce a mechanism for GEF-mediated nucleotide exchange. Also, the authors observe an interaction between the GEF-complex and the unstructured C-terminal half of Rab6, the so-called hypervariable domain (HVD). This suggests an interesting additional layer of recognition between GEF and Rab-GTPase. The authors test the importance of this HVD recognition for Rab6 activation by Ric1-Rgp1 in vivo utilizing an HVD-swap

mutant replacing the HVD of Rab6 by the Rab5-HVD. Furthermore, they propose a potential amphipathic helix in the subunit Rgp1. The authors describe this helix as important but not essential for correct cellular GEF localisation. Taken together the presented findings provide substantial new insights into the activation mechanism of the Rab6-GEF Ric1-Rgp1 and its cellular localization.

The study presented here is clearly written and the results are of high quality. The obtained data support the conclusions the authors make. The manuscript describes very interesting new insights deduced from the presented cryo-EM structure and tested in well-conceived biochemical and cellular experiments. These new insights will be of interest to a brought readership interested in Golgi organization, membrane trafficking and GEF-Rab interaction and activation.

Overall, the study of Feathers et al. is of very good quality, the rationale, the described results and the presentation of data are easy to follow and understand. However, some of the conclusions are based on a very thin layer of biochemical data and need to be addressed:

We thank the reviewer for their strong support of our study, and we have now provided additional biochemical data as described in our response to point #2 below.

We note that the yields of the Ric1-Rgp1 protein purifications are extremely low. As stated in the Methods section, the yield is only ~20 micrograms of Ric1-Rgp1 per 12 liters of cultured yeast. This situation makes it challenging to perform extensive biochemical experiments. Nevertheless, we agree with the Reviewer's suggestions to perform additional biochemical experiments, and we have now done so as detailed in Point 2 below. These results now demonstrate the importance of the interaction between the Rab6 HVD and Ric1-Rgp1.

1) The GEF assay presented in Figure 2 needs some additional controls. The authors should test for the intrinsic nucleotide exchange rate of Rab6 under these conditions. Is there remaining activity of the GEF-domain mutant or is the observed rate identical to the intrinsic exchange rate of Rab6 without GEF? In this context also a titration of the wild type vs. the mutant GEF

complex would be of interest. Is there residual activity of the mutant complex which would only be visible if higher amounts of the GEF were tested? In the context of the GEF assay, the authors also might want to control the membrane association of the GEF complex. Is it a loss of GEF activity or is there a problem with the membrane association of the mutant GEF-complex in the in vitro assay? However, the in vivo data suggests that the mutant GEF-complex is binding to membranes, but as the authors point out this might also be due to the action of Rab33 as a recruiter which is missing in the in vitro GEF assay.

We apologize for omitting this important control from the original figure. We did perform a “mock” (no GEF) control when we originally performed the experiment but did not include the data in the original figure panel because we mistakenly thought it would be clear that the mutant had no activity (we now realize we should have included the mock trace to make it more obvious). So now in the revised figure, the mock trace is included in Figure 2D, making it very clear that the mutant completely lacks GEF activity.

We have also now added the reaction conditions to the legend as requested.

We observed that the Ric1-Rgp1 complex does not bind stably to liposomes, providing further support for our idea that there is an unknown protein that serves to recruit Ric1-Rgp1 to the Golgi. Therefore it is currently not possible to test whether mutants interfere with membrane binding in vitro. Furthermore, given the location of F860 and R912 in the structure, in which they directly contact the nucleotide-binding domain of Rab6, we cannot imagine how these residues could be involved in membrane binding if their function is to interact with the nucleotide binding site.

We did not perform a titration of WT vs mutant GEF because our goals with these experiments were to validate the GEF-Rab interface seen in the structure and then to determine the consequences of a GEF-dead mutant in vivo. The GEF assay demonstrates the mutant has lost GEF activity, and the in vivo results demonstrate that loss of GEF activity is equivalent to a loss of function. We do not think a titration would tell us anything more about the nucleotide exchange mechanism, and such an experiment is simply not feasible given our extremely limited protein purification yields.

2) The in vivo experiments presented in Figure 4 suggest that “the chimeric GFP-Rab6-HVDRab5 was significantly mis-localized,...” (page 11, top paragraph) which was not leading to a growth defect at 38°C (Fig S3c). To narrow down the impact of the HVD-swap on Rab6 activation the authors should use their in vitro GEF-assay to measure activation rates of Ric1-Rgp1 towards wildtype and HVD-swap Rab6. This will allow the authors to get an idea of how much the HVD contributes to the GEF-Rab affinity.

To address this point and also Reviewer 4, Point 5, we have now further dissected the importance of the HVD using biochemical experiments, with interesting results. To do this we created new purification constructs for Rab6 mutants, and to avoid problems that could arise from challenges in performing the enzymatic prenylation reactions on the purified mutant constructs, we made these constructs with 7xHis-tags to anchor them to nickel-lipid containing liposomes. This also allowed us to test them as substrates in GEF assays that lack liposomes, which cannot be done with the prenylated substrates. We designed two mutants, one in which the CIM motif of the HVD is mutated (I200D/I202D), and another in which the HVD is swapped for that of Rab5. The results of these experiments are shown in new Fig. 4 g-j, described in a new section of the Results subtitled “The Rab6 HVD is important for activation via Ric1-Rgp1 catalyzed nucleotide exchange” and also summarized here:

Activation of the I200D/I202D mutant by Ric1-Rgp1 was significantly reduced compared to the wild-type, both in the presence and absence of liposomes. This provides convincing evidence that the interaction between Rgp1 and the Rab6 HVD observed in the cryoEM structure does contribute significant binding energy during the nucleotide exchange reaction.

The Rab5-HVD chimera was also a worse substrate than the wild-type, the difference was not as severe but was statistically significant.

We therefore conclude that the Rab6 HVD does indeed contribute meaningfully to Rab6 activation by Ric1-Rgp1, but that Ric1-Rgp1 does not necessarily use the HVD sequence to distinguish between Rab6 and other Rabs. We therefore made some small edits to the manuscript so that it is clear that we think the HVD is important for Rab6 activation and localization but is not necessarily a critical specificity factor used by Ric1-Rgp1 for

distinguishing between different Rabs. We think this is an important finding and we thank the Reviewer for suggesting the additional experiments.

3) Based on AlphaFold predictions the authors suggest the presence of an amphipathic helix. Deletion of the according amino acids leads to a loss of localization of Rgp1-GFP *in vivo*. However, they do not provide any evidence that this alpha helix has an amphipathic character. I would suggest presenting a helical wheel representation and calculating the hydrophobic moment of that helix. Additionally to the presented deletion, a mutational analysis of this helix should be included to test the hypothesis of an amphipathic helix and to verify the interpretation of the deletion mutant. Usually, the membrane association of proteins with potential amphipathic helices is also tested in membrane association assay, where the wildtype protein and mutant proteins are compared.

We have followed the reviewer's suggestion and now include a helical wheel in Figure 5e. We intended for the close-up view of the AlphaFold-predicted helix to serve as the evidence for its amphipathic nature, but we agree that the helical wheel provides additional important information.

We have demonstrated that the predicted amphipathic helix is required for normal Golgi localization *in vivo*, and this is the main point we are making. To further determine whether the helix is sufficient for membrane binding would require extensive additional investigations. In our previously published analysis of an amphipathic helix in Age2, Golgi localization of the protein was lost even when the helix was mutated to a sequence that maintained the amphipathic character (Manzer et al. MBoC 2023). This implies that the precise biophysical properties of particular amphipathic helix sequences can be critical determinants of membrane insertion in cells. It is also possible that the Age2 amphipathic helix binds to a Golgi protein rather than directly to lipids in cells. We therefore think a mutational analysis of the Rgp1 amphipathic helix may not necessarily add additional information other than what we already know, namely that this predicted amphipathic helix is important for Golgi localization in cells.

And as stated in our comments above, we are currently unable to assess membrane-binding of mutant Ric1-Rgp1 complexes *in vitro* because the wild-type protein does not bind stably to liposomes by itself, apparently requiring

another unknown recruiter protein to do so. This is similar to our recently published work on the Arf-GEF Sec7, which possesses an amphipathic helix that is important, but not essential, for Golgi localization. We therefore hope the Reviewer agrees that further characterization of the Ric1-Rgp1 membrane-binding mechanism is beyond the scope of the current work which already reports several major new findings.

4) Optionally the authors could also assess if the membrane association mediated by the amphipathic helix is necessary for the observed GEF-activity *in vitro* using again wildtype and mutant proteins. Since Rab33 is not present in their GEF-assay this would address if there are other membrane association cues in the Rab6 GEF-complex or, like the authors suggest in their discussion, the complex only relies on the suggested amphipathic helix and the interaction to Rab33.

As stated in the Discussion of the original submission, the Rab33 binding region of human Ric1 is not conserved in yeast Ric1, and we do not yet know exactly how yeast or human Ric1-Rgp1 is recruited to the Golgi. At this point we believe that additional *in vitro* experiments cannot tell us how Ric1-Rgp1 is recruited to the Golgi *in vivo* without additional screening. We are therefore planning to use *in vivo* proximity labeling (BioID) approaches to identify potential *in vivo* binding partners of Ric1-Rgp1. Once a potential recruiter of Ric1-Rgp1 is identified, then we can use *in vitro* approaches to dissect the relative contributions of protein-protein and protein-membrane interactions for Ric1-Rgp1 membrane association.

Reviewer #3 (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.

Reviewer #4 (Remarks to the Author):

The article by Feathers et al describes the molecular basis for how the Ric1-

Rgp1 complex activates the Rab6 GTPase. This is a highly conserved complex through evolution and mechanistic information of how it mediates nucleotide exchange is a useful contribution. The authors identified a previously uncharacterised helical domain that mediates GEF exchange, as well as an unexpected interaction of an arrestin fold of the GEF with the hypervariable domain of Rab6.

Overall this is clearly presented study that provides novel insight into an important GEF that regulates the Golgi complex. I have some suggestions that will be required to clarify and validate some of the analysis, and with this I am strongly supportive of publication in Nature Communications.

We thank the reviewer for their strong support of our study.

Major Revisions

1. Thank you to the authors for providing the map and pdb files for analysis. One of the things missing in the manuscript is the correlation between the map and the model, especially for the residues mediating the interface. I would strongly suggest showing a clear zoom in on the interface where the map is shown for this residues (essentially the zoom in shown in Fig 2C, except in this figures showing the map for these regions as a new supplemental figure). Essentially show the figure in S2D, but with a clear focus on the interfacial residues at the GEF domain.

We have now included a new figure (new Supplementary Figure S3) to address this important suggestion and a similar suggestion from Reviewer 1.

2. In addition it would also be very useful to see the sequence alignment of the helices in Ric1 that mediate GEF activity across evolution, as this would clearly additionally highlight the important role of F860 and R912. I recommend adding this as a panel to Figure 2.

We have now included a sequence alignment of this region of Ric1 as new figure panel Fig. 2g.

3. The authors claim that the F860A-R912A complex is catalytically dead. Do the authors know that the F860A-R912A complex is catalytically dead? Without the control experiment in panel 2D showing no GEF present, we can't determine if the GEF is dead (ie same activity as no GEF present). please rephrase or show the control experiment. Please include the experimental conditions for this assay in the figure legend of Fig 2D (concentrations [nucleotide, GEF, Rab, and membrane], and membrane composition) so readers don't need to chase this down in the methods.

We apologize for omitting this important control from the original figure. We did perform a "mock" (no GEF) control when we originally performed the experiment but did not include the data in the original figure panel because we mistakenly thought it would be clear that the mutant had no activity (we now realize we should have included the mock trace to make it more obvious). So now in the revised figure, the mock trace is included in Figure 2D, making it very clear that the mutant completely lacks GEF activity.

4. Have the authors tried a DALI search to see the closest fold to the helical domain of Ric1? As it does not share homology to other GEFs this could be useful information to include.

We did try DALI and Foldseek searches and the results don't seem very meaningful, as the top hits (other than likely Ric1 homologs in the AlphaFold database) are proteins of many different functions without any common theme. In case you are curious here are the top DALI hits of the Ric1 GEF domain against the PDB90:

No:	Chain	Z	rmsd	lali	nres	%id	PDB	Description
☐ 1:	6wlg-A	6.8	4.2	135	361	13	PDB	MOLECULE: INTEGRATOR COMPLEX SUBUNIT 3;
☐ 2:	6j6g-v	6.8	4.1	140	722	3	PDB	MOLECULE: PRE-MRNA-SPLICING FACTOR 8;
☐ 3:	8rbz-h	6.8	4.2	144	887	11	PDB	MOLECULE: UNK-UNK-UNK-UNK-UNK-UNK-UNK-UNK;
☐ 4:	8fbj-A	6.7	4.5	141	307	15	PDB	MOLECULE: KWOC_60;
☐ 5:	2p58-C	6.7	3.2	104	112	12	PDB	MOLECULE: PUTATIVE TYPE III SECRETION PROTEIN YSCE;
☐ 6:	6bk8-U	6.6	3.9	117	637	3	PDB	MOLECULE: U2 SNRNA;
☐ 7:	8hpp-A	6.5	4.4	140	395	11	PDB	MOLECULE: INTEGRATOR COMPLEX SUBUNIT 3;
☐ 8:	6e9y-A	6.4	4.7	134	227	8	PDB	MOLECULE: DHF38 FILAMENT;
☐ 9:	7wlm-D	6.4	4.2	136	967	12	PDB	MOLECULE: STRUCTURAL MAINTENANCE OF CHROMOSOMES PROTEIN 1A;
☐ 10:	7cun-H	6.3	3.9	142	912	10	PDB	MOLECULE: INTEGRATOR COMPLEX SUBUNIT 1;
☐ 11:	6e9z-A	6.3	4.7	137	225	9	PDB	MOLECULE: DHF119 FILAMENT;
☐ 12:	2uwj-G	6.2	3.2	101	113	11	PDB	MOLECULE: TYPE III EXPORT PROTEIN PSCE;
☐ 13:	6wg3-D	6.1	4.7	112	932	6	PDB	MOLECULE: STRUCTURAL MAINTENANCE OF CHROMOSOMES PROTEIN 1A;
☐ 14:	6k9k-A	6.0	4.1	129	2501	6	PDB	MOLECULE: SERINE-PROTEIN KINASE ATM;
☐ 15:	1h2u-A	6.0	5.2	124	705	10	PDB	MOLECULE: 80 KDA NUCLEAR CAP BINDING PROTEIN;
☐ 16:	6xr1-A	6.0	4.3	143	513	10	PDB	MOLECULE: DTOR_9X57R;

5. The discovery of the arrestin fold in Rgp1 binding to the HVT is an intriguing discovery. It would be useful for the authors to mutate residues in the HVT and test their in vitro GEF assay. This would likely have to be done in the absence of prenylation, as it would also disrupt this. Does the Ric-RGP complex still have GEF activity in the absence of membrane? If so it would be worth trying GEF assays with and without the mutated HVT as a proof of this altering GEF activity. The cell experiments are nice, but may be working through a differential mechanism.

To address this point and also Reviewer 2's point 2, we have now further dissected the importance of the HVD using biochemical experiments, with interesting results. As suggested by the reviewer, we avoided prenylation by instead utilizing a C-terminal 7xHis-tag for anchoring to nickel-lipid containing liposomes. This also allowed us to test the importance of the HVD in GEF assays that lack liposomes, which cannot be performed with the prenylated Rab substrates.

We designed two mutants, one in which the CIM motif of the HVD is mutated (I200D/I202D), and another in which the HVD is swapped for that of Rab5. The results of these experiments are shown in new Fig. 4g-j, described in a new section of the Results subtitled "The Rab6 HVD is important for activation via Ric1-Rgp1 catalyzed nucleotide exchange" and summarized here:

Activation of the I200D/I202D mutant by Ric1-Rgp1 was significantly reduced compared to the wild-type, both in the presence and absence of liposomes. This provides convincing evidence that the interaction between Rgp1 and the Rab6 HVD observed in the cryoEM structure does contribute significant binding energy during the nucleotide exchange reaction.

The Rab5-HVD chimera was also a worse substrate than the wild-type, the difference was not as severe but was statistically significant.

We therefore conclude that the Rab6 HVD does indeed contribute meaningfully to Rab6 activation by Ric1-Rgp1, but that Ric1-Rgp1 does not necessarily use the HVD sequence to distinguish between Rab6 and other Rabs. We therefore made some small edits to the manuscript so that it is clear that we think the HVD is important for Rab6 activation and localization

but is not necessarily a critical specificity factor used by Ric1-Rgp1 for distinguishing between different Rabs. We think this is an important finding and we thank the Reviewer for suggesting the additional experiments.

6. It would also be useful to to show the sequence conservation of the interacting residues in the HVT in Fig4, instead of in the supplement as this is something that I think warrants being in the main figures.

Thank you for this suggestion. We have moved that panel to main Fig. 4d.

7. It would be useful for the authors to redo their alphafold multimer search with recently released alphafold3.

We are sorry that we do not know what the reviewer is referring to, as we did not perform an AlphaFold-multimer search or present any predictions generated using AlphaFold-multimer. We think the reviewer might be referring to the Rgp1 AlphaFold prediction we present in Figure 5d, so we examined the prediction of Rgp1 produced by the new AlphaFold3 server and found that it is essentially the same as the AlphaFold2 prediction shown in Figure 5d. We have therefore not changed that figure panel.

Minor revision.

1. The GEF assay shown in Fig 2D is very hard to read with fonts being extremely small, please improve the clarity of this figure.

Thank you for pointing this out, we have increased the font size in the revised figure.