# RGS4 controls Gαi3-mediated regulation of Bcl-2 phosphorylation on TGN38-containing intracellular membranes

Guillaume Bastin, Kaveesh Dissanayake, Dylan Langburt, Alex L.C. Tam, Shin-Haw Lee, Karanjit Lachhar and Scott P. Heximer
DOI: 10.1242/jcs.241034

**Editor**: John Heath

**Review timeline**

| | |
|---|---|
| Original submission: | 30 October 2019 |
| Editorial decision: | 6 December 2019 |
| First revision received: | 16 January 2020 |
| Editorial decision: | 10 February 2020 |
| Second revision received: | 19 February 2020 |
| Accepted: | 5 May 2020 |

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**Original submission**

First decision letter

MS ID#: JOCES/2019/241034

MS TITLE: RGS4 controls Gαi3-mediated regulation of Bcl-2 phosphorylation on TGN38-containing intracellular membranes

AUTHORS: Guillaume Bastin, Kaveesh Dissanayake, Dylan Langburt, Alex Tam, Shin-Haw Lee, Karanjit Lachhar, and Scott Heximer
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

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

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

Reviewer 1

*Advance summary and potential significance to field*

Bastin et al. report that the heterotrimeric G-protein alpha subunit Gαi3, previously inferred to inhibit JNK activation at TGN38-labelled vesicles, exhibits similar requirements for inhibiting phosphorylation of the established JNK target, Bcl-2.
This has predictable effects to inhibit nutrient deprivation-induced autophagy.

*Comments for the author*

1. Fig. 1A appears to be mislabeled, including legend. The two conditions represent WT and RC alleles of Gαi3 expressed in Gαi3-KO cells.

2. In Fig. 1B, does JNK activity differ between Gαi3-WT and Gαi3-RC cell lines as a potential explanation of the heightened phosphor-Bcl-2 levels in RGS4-overexpressing cells?

3. In Fig. 1C and line 148-151, the lack of efficacy for RSG4, a GTPase activator, in cells expressing a GTPase-dead version of Gαi3 is unsurprising.

4. The images in Fig. 5 are not convincing in demonstrating the effect of DHHS7 in preventing RSG4 localization to TGN38-positive vesicles (would be clearer if RSG4-YFP was presented as yellow rather than green pseudocolor or choosing a colocalization threshold mask of a different pseudocolor).

5. Fig. 5C requires additional explanation. How many endosomes/cells are represented for each point? How were endosome diameters (a continuous variable) binned for representation of a collection of endosomes? How were multiple pixels for individual endosomes combined to calculate pixel intensity? The results appear consistent with a smaller distribution of endosome sizes in DHHS3 and DHHS7-expressing cells. The conclusion that RSG4 is expressed on TGN38-negative endosomes is not apparent from the scatterplots (are all endosome sizes with negative PCC values RSG4-positive, TGN38-negative or a mixture of single positives?). How are RSG4-positive, TGN38-negative bodies identified as endosomes?

6. In Fig. 5B, the colocalization of RGS4 and TGN38 appears similar between control and DSSH3-expressing cells, suggesting that Gαi3 interactions with its inhibitor RSG4 on TGN38-labeled endosomes will be equivalent. If so, why does DSSH3 expression provide greater inhibition of Gαi3-dependent inhibition of JNK (reported by Bcl-2 phosphorylation), compared to control cells. Are there differences in the localization of Gαi3, JNK or Bcl-2 to the TGN38-labeled compartment?

7. Why is the initial chase (1 hr) for degradation of short-lived proteins carried out in nutrient-depleted media? How does Gαi3-RC and RSG4 transfection affect proteosomal turnover (+/- proteasome inhibitor)?

Reviewer 2

*Advance summary and potential significance to field*

The authors investigate signaling of the heterotrimeric G-protein alpha subunit, Gαi3. The study suggests a link between Gαi3 activity and decreased Bcl-2 phosphorylation. Altered autophagic signaling in response to changes in the localization of the Gαi3 inhibitor RGS4 let the authors to conclude a regulatory role of TGN38-labelled vesicle pools. While the presented results and the suggested signaling pathway are interesting, a few concerns remain.

*Comments for the author*

1) The authors chose to perform the experiments with overexpressed BCL-2.

Massive overexpression could shift protein pools within the cell and thus vastly increase the pool available for phosphorylation. The authors would strengthen their case with experiments analyzing endogenous BCL-2.
2) Along the same lines DHHC localizations could be impaired by overexpression.
3) Are the presented effects direct or simply indicating altered cell stress signaling? The characterization of transient or stable protein interactions would help to appreciate direct and specific signaling.
4) The authors stress the importance of proteins in TGN38-labelled vesicle pools. Is BCL-2 or JNK present in these vesicles?

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**First revision**

Author response to reviewers' comments

Response to Reviewers:

Reviewer 1 Comments for the author
1. Fig. 1A appears to be mislabeled, including legend. The two conditions represent WT and RC alleles of Gαi3 expressed in Gαi3-KO cells.

Thank you for your comment. Yes, we have corrected Figure 1 labels and legend to indicate that experiments were performed in Gαi3-KO cells expressing WT and RC alleles. The legend text now reads: "A, Western blots (upper panel) for phospho(P)-Bcl2 and total(T)-Bcl2 levels in CRISPR-Gαi3 -KO HEK293 cells transiently expressing either a WT or RC allele of Gαi3. Histogram (lower panel) shows quantitation of the P-Bcl2:T-Bcl2 ratios." We appreciated your attention on this, we are very sorry for this mistake, thank you!

2. In Fig. 1B, does JNK activity differ between Gαi3-WT and Gαi3-RC cell lines as a potential explanation of the heightened phosphor-Bcl-2 levels in RGS4-overexpressing cells?

Indeed, we believe differences in Gαi3, and as a result JNK, activity between the different conditions are a key determinant of the pBcl-2 levels. In our previous manuscript entitled : "Gαi3-Dependent Inhibition of JNK Activity on Intracellular Membranes.", the regulation of JNK by Gαi3 -WT and -RC was tested and -RC was shown to decrease the levels of P-JNK under similar condition of nutrient starvation as we here report here for P-Bcl2 regulation. Within the same study, we showed that RGS4 could also regulate P-JNK, but interestingly only the intracellular pool of RGS4 (RGS4-C12A which is specifically targeting TGN38) increased the level of P-JNK. These data provided the rationale for us to test of the activity of both Gαi3 and RGS4 on the regulation of p-Bcl2 levels since both JNK and Gαi3 were previously reported to regulate autophagy. Our study is the first to connect all of these previously independent unlinked stories.

To help clarify this for the reader, we have added the citation to our previously published study to the paragraph, line 136. (the text has been highlighted in yellow accordingly as requested by revision guidelines of JCS).

3. In Fig. 1C and line 148-151, the lack of efficacy for RSG4, a GTPase activator, in cells expressing a GTPase-dead version of Gαi3 is unsurprising.

We are sorry if our statement was not clear. Traditionally, the Q204L GTPase-deficient mutants of heterotrimeric G-proteins have been used as constitutively active mutant because it is locked in the GTP-bound (activated) state where they can interact with downstream effectors and cannot recouple to Gβγ heterodimer or GPCRs. The surprising nature of the pBcl2 phenotype for us was that it did not seem to depend on the activated conformational state of Gαi3, but rather the extent of GTPase cycling implying a highly dynamic component for this pathway.

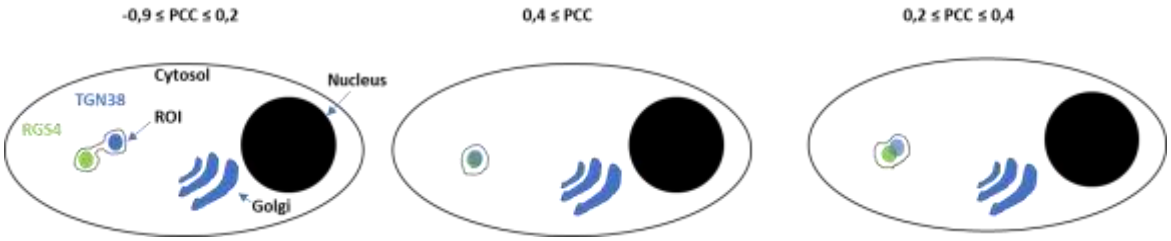
To hopefully better clarify this aspect, this paragraph in the results was reorganized (lines 142-159). And the paragraph subheading was added as followed: "Gαi3 GTPase activity enhanced cycling by RGS4 increases the phosphorylation status of Bcl-2." (the text has been highlighted in yellow accordingly as requested by revision guidelines of JCS).

4. The images in Fig. 5 are not convincing in demonstrating the effect of DHHS7 in preventing RSG4 localization to TGN38-positive vesicles (would be clearer if RSG4-YFP was presented as yellow rather than green pseudocolor or choosing a colocalization threshold mask of a different pseudocolor).

Thank you for your feedback. To overcome the issue, pictures were re-collected using another confocal microscope so that the colors show up better, Panel A of figure 5 was remade accordingly. Arrows also have been placed to guide the reader. We are hoping that it is now looking more convincing.

5. Fig. 5C requires additional explanation. How many endosomes/cells are represented for each point? How were endosome diameters (a continuous variable) binned for representation of a collection of endosomes? How were multiple pixels for individual endosomes combined to calculate pixel intensity? The results appear consistent with a smaller distribution of endosome sizes in DHHS3 and DHHS7-
expressing cells. The conclusion that RSG4 is expressed on TGN38-negative endosomes is not apparent from the scatterplots (are all endosome sizes with negative PCC values RSG4-positive, TGN38-negative or a mixture of single positives?). How are RSG4-positive, TGN38-negative bodies identified as endosomes?

We are sorry this was not clear. Each data point in figure 5C represents one endosome positive for RGS4-YFP. At baseline, without the DHHS constructs, about 40% of the cells show one or two RGS4-YFP punctae (from Figure 4). For each individual RGS4-YFP containing endosome we measured its diameter using the FluoView A-1000 software package from Olympus. The FluoView A-1000 software also allowed us to select individual endosomes as regions of interest/ROI and obtain PCC data for each RGS4-YFP containing endosome. Examples of the different expected ranges of PCC data for RGS4-YFP containing endosomes with TGN38 are provided in the cartoon below.




To improve the clarity of the strategy the following sentence was added to the Materials and Method section line 469: "regions of interest containing RGS4-YFP endosomes were manually selected to quantify the PCC between each RGS4 and TGN38 endosomes (not as whole cell/field)." (the text has been highlighted in yellow accordingly as requested by revision guidelines of JCS).

We used this strategy because TGN38 is more prevalent in the Golgi compared to the endosomal pool. Therefore, if we were to consider whole cells and compare their co-localization, the PCC would not be representative of what happened outside of the core of the Golgi. Please note that this strategy was previously published: "Bastin et al. 2012. Amino-terminal cysteine residues differentially influence RGS4 protein plasma membrane targeting, intracellular trafficking, and function."

Using this approach, it became very clear that DHHS7 decreased the co-localization between RGS4 and TGN38.

In this manuscript, we do not specifically show that RGS4-containing intracellular membranes are endosomes, however, TGN38 is known to traffic between the TGN and the plasma membrane via endosomes, and RGS4 has been previously shown by our group and others to target intracellular endosomal pools: Brandon M Sullivan et al. 2000: "RGS4 and RGS2 Bind Coatomer and Inhibit COPI Association with Golgi Membranes and Intracellular Transport." and Guillaume Bastin et al, 2013: "Rab family proteins regulate the endosomal trafficking and function of RGS4."

6. In Fig. 5B, the colocalization of RGS4 and TGN38 appears similar between control and DSSH3-expressing cells, suggesting that Gαi3 interactions with its inhibitor RSG4 on TGN38-labeled endosomes will be equivalent. If so, why does DSSH3 expression provide greater inhibition of Gαi3-dependent inhibition of JNK
(reported by Bcl-2 phosphorylation), compared to control cells. Are there differences in the localization of Gαi3, JNK or Bcl-2 to the TGN38-labeled compartment?

This is an excellent question. Indeed, DHHS3 does not change the co-localization between intracellular RGS4 and TGN38 endosomes, however DHHS3 changes the prevalence of RGS4 in intracellular compartment (Figure 4C). Notably, DHHS3 greatly increases the frequency of cells showing RGS4 on intracellular membranes. Because we isolate RGS4-YFP endosomes as ROIs (see discussion for point 5 above), we would not necessarily expect an increase in PCC under these circumstances. Therefore, the phenotype related to RGS4 function on intracellular pools positive for TGN38 is best demonstrated by more global cell readouts such as the Western blotting data in Figure 6.

Line 214-216 were modified to help clarity this point. Thank you for this review. (the text has been highlighted in yellow accordingly as requested by revision guidelines of JCS).

7. Why is the initial chase (1 hr) for degradation of short-lived proteins carried out in nutrient-depleted media? How does Gαi3-RC and RSG4 transfection affect proteosomal turnover (+/- proteasome inhibitor)?

These are good questions. This protocol was developed by Patrice Codogno, a leader in the autophagy field who first uncovered the role of Gαi3 as one of its regulators. I was in direct contact with him while we established these assays in our lab. The Codogno protocols focus on "long-lived" protein degradation as the primary mode of their catabolism under starvation conditions which is believed to be autophagy-mediated.

The use of 3-MA, a relatively selective inhibitor of autophagy initiation, provides some insight into the extent of "long-lived" protein degradation linked to autophagosomal degradation. Although, it is true that a portion of the 14C-leucine labelled protein degradation is not blocked by 3-MA addition (Fig. 7, 8B and S7), when autophagy is blocked (3-MA addition) the protein degradation rates are very similar between RGS4 WT and EN-AA mutant conditions, leading us to conclude that RGS4 and Gαi3 preferentially regulate autophagy rather than proteasomal mediated protein degradation.

In line 231, the following sentence was extended: "3-MA eliminated the differences flux between RGS4-ENAA and RGS4-WT in this assay, this data supports that RGS4 activity regulates 14C-leucine labelled protein degradation mainly through the regulation of autophagy." (the text has been highlighted in yellow accordingly as requested by revision guidelines of JCS).

We would like to thank you for your feedback and for the careful attention you provided to this manuscript, we believe that your reviews have greatly improved the quality of this work.

Reviewer 2 Comments for the author
1) The authors chose to perform the experiments with overexpressed BCL-2. Massive overexpression could shift protein pools within the cell and thus vastly increase the pool available for phosphorylation. The authors would strengthen their case with experiments analyzing endogenous BCL-2.

Thank you for your important comment on the Bcl-2 expression levels. We acknowledge your concerns regarding possible overexpression artefacts in our system. Because attempts to detect total and phospho-Bcl2 in our cell model were unsuccessful (even following immunoprecipitation), we contacted a local expert in the Bcl-2 field for help and advice (Dr. David Andrews, Sunnybrook Hospital, Toronto). Dr. Andrews provided us with an expression construct and detailed instructions for titration of the plasmid to prevent the type of massive overexpression artefacts you are referring to. In our system, the Bcl-2 levels were just high enough for detection by Western blotting but without causing any deleterious effects on cell growth and viability.

A sentence was added to the material and method section, line 415: “the amount of Bcl2 was carefully titrated to allow its detection while avoiding any deleterious effects on cell growth and viability.” (the text has been highlighted in yellow accordingly as requested by revision guidelines of JCS).

2) Along the same lines DHHC localizations could be impaired by overexpression.

Indeed, it is also possible that overexpression of a DHHC enzyme could affect its localization. There is a study showing that endogenous DHHC3 and over expressed HA-DHHC3 are statistically differentially co-localizing with cis-Golgi and trans-Golgi markers in HEK293 cells, nevertheless the Golgi compartments targeted by DHHC3 remain consistent and the distribution remains largely representative of the endogenous protein, figure 7G and 7H of the study: Casey L. Kilpatrick et al. 2016, JBC, VOL. 291, NO. 53, pp. 27371–27386: Dissociation of Golgi-associated DHHC-type Zinc Finger Protein (GODZ)- and Sertoli Cell Gene with a Zinc Finger Domain- (SERZ-)-mediated Palmitoylation by Loss of Function Analyses in Knock-out Mice. (DHHC3 being also called GODZ).

The DHHC/S clones in our study were used mainly as genetic tools (dominant negative (DHHS) and control isoforms) to inhibit endogenous DHHC function and affect RGS4 localization. Thus their overexpression was needed to help us to correlate RGS4 localization with its function as a regulator of G$\alpha$i3 signaling, Bcl2 phosphorylation and autophagy. Interestingly, the data were very consistent with our previous work showing that G$\alpha$i3 and RGS4 activity could regulate endogenous JNK activity from within TGN38 positive pools.

3) Are the presented effects direct or simply indicating altered cell stress signaling? The characterization of transient or stable protein interactions would help to appreciate direct and specific signaling.

We presume that the primary “stress” signaling pathways are those that are activated by nutrient-deprivation and have been previously linked to upregulation of autophagic flux. As discussed in our introduction, Patrice Codogno has already shown that G$\alpha$i3 signaling can modulate these signaling pathways. We here endeavor to provide a more complete mechanistic explanation for some of the autophagy-regulating pathways with which G$\alpha$i3 intersects.

As far as transient versus stable interactions are concerned, this is an excellent question. The data in figure 1 B and C show that the cycling of G$\alpha$i3-GTP to G$\alpha$i3-GDP is crucial for RGS4 to increase P-Bcl2 through G$\alpha$i3. We believe this shows that we are looking at a very dynamic/transient interaction and not a steady on or off signaling state. A paragraph in the discussion addresses the potential signaling pathways that may be involved in the regulation of Bcl2 via G$\alpha$i3 cycling (line 291-315).

To extend the discussion on the presumed transient aspect of these interactions, we added the following statements (line 310-315): “The data herein showed that the cycling of G$\alpha$i3 from GTP- to GDP-bound states was a major determinant of the regulation of Bcl-2 phosphorylation status by RGS4. These data would therefore suggest that regulation of Bcl-2 requires a transient interaction with G$\alpha$i3 partner(s)/effector(s) similar to that which is normally used by other small GTPases such as Arf1 to mediate membrane trafficking events. It will, therefore, be of interest to determine whether G$\alpha$i3 and Arf1 show overlapping functions as regulation of autophagy.” (the text has been highlighted in yellow accordingly as requested by revision guidelines of JCS).

The paragraph 142-159 was also reorganized to emphasize the implication of the GTPase cycling rather than the traditional active or inactive state of Gα proteins. (the text has been highlighted in yellow accordingly as requested by revision guidelines of JCS).

4)The authors stress the importance of proteins in TGN38-labelled vesicle pools. Is BCL-2 or JNK present in these vesicles?

Our current and previous data would support a model where Bcl-2 and JNK are in the vicinity of TGN38/RGS4/Gαi3-containing vesicles, but with the variety of known effectors for Gαi3 (Discussion line 291-315), regulate JNK and Bcl-2 via an indirect mechanism. One important reason for this assumption is because activated Gαi on the plasma membrane appears to activate JNK whereas activated Gαi on TGN38-containing pools appears to inhibit JNK activation. Thus, it appears there are different effector pathways for activated Gαi3 within different compartments of the cell that indirectly mediate the effects of its activation (Bastin et al. 2015).

The following statement was added to the discussion (line 323-325) to raise this point to the attention of the readership: “The differential regulation of JNK and Bcl-2 by RGS4 and Gαi3 between these different intracellular compartments suggests that different Gαi3 effectors are involved.” (the text has been highlighted in yellow accordingly as requested by revision guidelines of JCS).

Overall, we would like to thank you very much for your valued comments and questions. Their consideration has greatly improved the quality of this work.

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Second decision letter

MS ID#: JOCES/2019/241034

MS TITLE: RGS4 controls Gαi3-mediated regulation of Bcl-2 phosphorylation on TGN38-containing intracellular membranes

AUTHORS: Guillaume Bastin, Kaveesh Dissanayake, Dylan Langburt, Alex L.C. Tam, Shin-Haw Lee, Karanjit Lachhar, and Scott P. Heximer
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers gave favourable reports but raised some critical points that will require editorial amendments to the text of your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

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

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

Reviewer 1

*Advance summary and potential significance to field*

The authors have addressed some of the concerns with the manuscript.

*Comments for the author*

However, the following issues still remain:

1. The authors conclude that Gαi3 function is linked to GTPase activity, rather than the GTP bound state, based on the differential response of two mutants of Gαi3 one with GAP-regulated GTPase activity and one with defective GTPase activity, to the GAP protein RGS4. The existence of these mutants themselves argues against their interpretation, since both have low GTPase activity, but are constitutively active. Co-expression of RGS4 appropriately increases P-Bcl-2 levels (inhibiting Gαi3, a negative regulator of the Bcl-2 JNK kinases) for the RC Gαi3 mutant, and as expected, does not increase P-Bcl-2 levels for the QL Gαi3 mutant, since Gαi3-GTP levels are not affected by GAP expression. Additionally, references for the functional validation of the RC/QL mutants should be provided.

2. The differential effect of dominant negative acyltransferases DHHS3 and DHHS7 on RSG4 stimulated Bcl-2 phosphorylation (shown in Fig. 6), is the central evidence for the localization of the Gαi3-JNK-Bcl-2 pathway to TGN38-positive endosomes.
This should also be compared to controls without DHHS3/7 expression. Since the number of cells with endosomal localization of RSG4 is approximately double in the DHHS3-expressing cells, there should be a commensurate increase in P-Bcl-2.

Reviewer 2

*Advance summary and potential significance to field*

Please see below.

*Comments for the author*

The authors have adequately addressed my concerns with the limitation that experiments are exclusively performed with ectopically expressed protein.
However, the reader is sufficiently informed about this limitation.

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**Second revision**

Author response to reviewers' comments

1.The authors conclude that Gαi3 function is linked to GTPase activity, rather thanthe GTP bound state, based on the differential response of two mutants of Gαi3, one with GAP-regulated GTPase activity and one with defective GTPase activity, to the GAP protein RGS4. The existence of these mutants themselves argues against their interpretation, since both have low GTPase activity, but are constitutively active. Co-expression of RGS4 appropriately increases P-Bcl-2 levels (inhibiting Gαi3, a negative regulator of the Bcl-2 JNK kinases) for the RC Gαi3 mutant, and as expected, does not increase P-Bcl-2 levels for the QL Gαi3 mutant, since Gαi3- GTP levels are not affected by GAP expression. Additionally, references for the functional validation of the RC/QL mutants should be provided.

We appreciate the reviewer’s concern regarding our conclusion that the observed effects were driven by GTPase cycling of Gαi3 rather than its conformational state (GTP- or GDP-bound). Our

contention was based on the following two assumptions: 1) RGS proteins form stable complexes with Gαi (Q204L) mutants in co-expression studies (Hunt et al., 1996-Nature; Dulin et al., 1999-Mol. Cell Biol.); and 2) RGS4 binding to Gαi3 (Q204L) is expected to inhibit Gαi3 (Q204L)'s interaction with downstream effectors of P-Bcl2 level regulation.This latter assumption is based on the known structural overlap of the RGS protein binding footprint on Gαi3 with the binding footprint used by many of the known effectors of activated Gα subunits - specifically the switch II/alpha helix 3 groove (Sprang, 2016- Biopolymers). This activity is known as the "effector-blocking" function of RGS proteins. We do, however, acknowledge the possibility that the Gαi3 effector interaction domain for the P-Bcl2 pathway may be novel, and that RGS4 binding to Gαi3 (Q204L) may not necessarily be expected to block this interaction. As a result, we have changed the paragraph in the results section to directly address the reviewer's concerns and are grateful for the opportunity to improve the quality of the manuscript. As requested, we have also included references to the original papers that characterized the constitutively active G-protein mutations and their regulation by RGS protein function.

Accordingly lines 142 to 158 in the RESULTS section now read:
Co-expression of wild type RGS4 (WT), a potent Gαi3 inhibitor, increased the level of P-Bcl-2 compared to a catalytically dead RGS4 (EN-AA) isoform. (Fig. 1B). The effect of RGS4 was greatest when it was co-expressed with a GAP-sensitive (Berman et al., 1996) constitutively-active Gαi3-RC construct (Coleman et al., 1994), consistent with the notion that either the activation state (i.e. GTP- or GDP-bound) or GTPase cycling function of Gαi3 is an important regulator of P-Bcl-2 levels in our system. AGS3, an inhibitor of Gαi3 activation that has been localized to ER and Golgi membranes (Oner, Vural and Lanier, 2013), also promoted increased
levels of P-Bcl-2 to the same extent as RGS4 (Fig. S2). Moreover, the P-Bcl-2-elevating effects of RGS4 could be prevented by either mutation of the RGS domain to the Gq-selective "triple-mutant" (RGS4-3x) form or co-expression of a RGS-insensitive mutant of Gαi3 (Gαi3-GS; Fig. S2). Surprisingly, P-Bcl-2 levels were not increased when RGS4 was co-expressed with a non-cycling GAP-resistant (Berman et al., 1996) constitutively active mutant of Gαi3, Gαi3-QL (Coleman et al., 1994, Sondek et al., 1994) (Fig. 1C). Since, RGS proteins form stable complexes with Gα-QL isoforms (presumably GTP-bound) in mammalian expression systems (Hunt et al., 1996, Dulin et al., 1999), we conclude that either: i) the P-Bcl2 inhibiting effects of GTP-bound Gαi3 are not affected by RGS4 binding; or ii) Gαi3 cycling between GTP- and GDP-bound states is required for its regulation of this pathway.

2.The differential effect of dominant negative acyltransferases DHHS3 and DHHS7on RSG4 stimulated Bcl-2 phosphorylation (shown in Fig. 6), is the central evidence for the localization of the Gαi3-JNK-Bcl-2 pathway to TGN38-positive endosomes. This should also be compared to controls without DHHS3/7 expression. Since the number of cells with endosomal localization of RSG4 is approximately double in the DHHS3-expressing cells, there should be a commensurate increase in P-Bcl-2.

This is a very good point. We agree that the vector control data in Figure 6 would help to support some of the earlier observations regarding the prevalence of RGS4-containing endosomes and is, therefore, useful to include. As predicted by the reviewer, DHHS3 significantly increased P-Bcl2 mediated by RGS4 as compared to empty vector. This data highlights the extent to which the prevalence of RGS4 on TGN-38-positive endosomes affects P-Bcl2 levels. Although, both DHHS3 (90%) and DHHS7 (90 %) roughly double the % of cells containing endosomes compared to control vector (45%) (Figure 4), the percentage of those endosomes colocalizing with TGN38 is higher for DHHS3 (58%) and control (64%) compared to DHHS7 (36%) (Figure 5). Assuming similar endosome numbers/cell, these ratios would predict that P-Bcl2 levels for the samples would be DHHS3 >> DHHS7 > vector control- this is exactly what is observed in Figure 6.

Overall, we are grateful for the comments of the reviewer and their careful consideration of some of the more nuanced aspects of this study. A revised Figure 6 that included the vector controls replaced the previous simplified Figure.
(the sections of manuscript text that have been changed are highlighted in yellow as requested by JCS revision guidelines)

Third decision letter

MS ID#: JOCES/2019/241034

MS TITLE: RGS4 controls Gαi3-mediated regulation of Bcl-2 phosphorylation on TGN38-containing intracellular membranes

AUTHORS: Guillaume Bastin, Kaveesh Dissanayake, Dylan Langburt, Alex L.C. Tam, Shin-haw Lee, Karanjit Lachhar, and Scott P. Heximer
ARTICLE TYPE: Research Article

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

Reviewer 2

*Advance summary and potential significance to field*

In my view, the manuscript can be published as it is.

*Comments for the author*

The authors have addressed my concerns with their previous revised version.