

The VPS33B-VPS16B protein complex facilitates Golgi ribbon formation and Golgi-endosome trafficking

Corresponding Author: Professor Walter Kahr

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 is a review of the manuscript titled "The VPS33B-VPS16B protein complex facilitates Golgi ribbon formation and Golgi-endosome trafficking" by Yao and co-workers. This work studies cellular defects in ARC syndrome, a severe disease caused by mutation of either VPS33B or VPS16B, proteins that associate into a tight complex. Significant previous knowledge on this topic has come from this research group. An important advancement presented here is the analysis of patient fibroblasts showing Golgi morphology defects both by electron and fluorescence microscopy (Fig. 1), extending previously recognized endo-lysosomal defects as well as deficiency in specialized organelles such as platelet granules. The study also provides nice new information on the endo-lysosome defects caused by ARC (for instance Fig. 7). However, other aspects of the manuscript appear somewhat preliminary as detailed below in the specific comments. Additionally, a previous VPS33B-Bio-ID screening detected VPS51 and VPS53 (Hunter et al, 2018, J. Mol. Biol.). This article needs to be appropriately cited, and the results compared with data presented here. Moreover, as discussed by Hunter et al, VPS51 and VPS53 not only are components of the GARP complex, which mediates endosome-to-Golgi transport, but also the EARP complex, which mediates endosome-to-cell surface transport. The data is interpreted here as if only GARP could be a VPS33B-VPS16B partner when it could be either one of them or both, and the data presented here does not clearly distinguish between these possibilities.

Major points:

1. Fig. 2: validation of the anti-VPS16B antibody needs to be shown. The Rab8 colocalization is significant and this Rab proteins is involved in transport to the cell surface. It would be important to use other markers of tubular endosomes like Rab4, STX12, retromer, or retriever. Comparison with GARP/EARP would be important also based on results presented later in the manuscript.
2. Fig 3. panels D and E: The staining pattern and localization of Myc-VPS16B is different from that of immunostained VPS16B (Fig. 2). Myc-VPS16B does not colocalize with the Golgi marker (Golgin-97). These data are not consistent with Figure 2.
3. Figure 4C: it would be important to perform co-IP of endogenous proteins, using the anti-VPS16B antibodies for the IP step and the VPS16B-KO HEK293 cells (or patient fibroblasts) as control. Additionally, in Fig. 4C (co-IP data with overexpressed proteins) it would help to have as an additional control a sample not expressing VPS33B-FLAG that is subjected to FLAG-IP to better control for GFP-GRASP55 non-specifically pulled down by FLAG-IP.
4. Fig. 5A: as mentioned above, the same Bio-ID screening was previously reported by Hunter et al (2018, J. Mol. Biol). This publication and the fact that it also detected VPS51/53 should be mentioned and discussed. The Hunter paper used performed parallel VPS33A Bio-ID for comparison/ control. What control was used in the current study? The possibility that VPS51/53 detected here are part of EARP complex needs to be incorporated or better yet experimentally test if it is GARP and not EARP (for instance by knocking down/out the specific subunit that distinguish each complex).
5. Fig 5B and C: Same comments as point #3 (co-IP of endogenous proteins, controls).
6. Fig 5I: Does Myc-VPS53 colocalize with VPS16B?
7. Line 261-264. While Magic red is a useful tool to study CatB, it really measures activity rather than the amount of the protein/enzyme per se. The activity of CatB can be affected by other factors like pH and the activity of other proteases that proteolytically activate it. To establish if there is less total catB, it would be important to analyze CatB by immunoblotting of cell extracts. This would also reveal if its processing is affected (different states, pre-protein, immature, mature can be identified by their size).
8. Fig. 7F should be quantified.

Minor points:

1. Affiliation # 7 for last author is not described
2. Fig. 1C and many other figures showing cells: it is difficult to visualize the cell edge. Please indicate the cell contour for instance using a dotted line.
3. Line 159-160: "We conclude from these observations that VPS33B-VPS16B is not required for Golgi stack formation, instead likely plays a role in the formation and/or stability of Golgi ribbons." It is not clear how the authors derive this conclusion, at least at this point of the manuscript.
4. Line 186: Figure 4F should be called/indicated.
5. Lines 213-218. The meaning of this sentence needs to be clearer or perhaps move it to the discussion section.
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Reviewer #2

(Remarks to the Author)

Yao et al explore the function of the VPS33B-VPS16B complex. They use patient fibroblasts from ARC syndrome and observe that there is lower expression of both members of the complex, and the Golgi apparatus is structurally abnormal. They then localise VPS16B to the Golgi apparatus. They make and validate a VPS16B KO in which the phenotype appears similar to the ARC fibroblasts, and they complement the phenotype with overexpressed VPS16B. They show an interaction through co-IP with GRASP55 (a intergolgi tether) and VPS33B, and overexpression of GRASP55 seemed to recover the Golgi morphology. They then perform BIOID with VPS16B, and identify the GARP complex components, which they also show interact by co-IP. There was a minor decrease in the co-localisation of TGN46 and VPS53 in the APC cells. VPS16B was partially mislocalised in VPS53 DN cells.

CIMPR trafficking was assessed through localisation of the receptor which was mislocalised in the APC cells, and it's ligand the lysosomal hydrolase was also less active. Finally, large endosomal like organelles were assessed as they were observed in TEM data, and they were shown to be properly acidified. Taken together, the authors present a model in which the VPS33B-VPS16B complex interacts with multiple Golgi machineries to modulate endosomal recycling and Golgi structure, which gives insights into disease pathology. The experiments are well performed and interpreted for the most part and the data clear and appropriately analysed.

Despite these positive points, I have some concerns regarding the depth of mechanistic insight and the directness of all the findings. In short, the new interaction partners were validated by IP from cells, so may be indirect and there was no biochemistry or validation to demonstrate this. Secondly, even if the interactions valid, the observations would be consistent with another model where VPS33B-VPS16B are doing something specific to the Golgi that indeed affects similar things to GARP and GRASP55. The best evidence of directness is the overexpression of GRASP55 on the complementation of the 16B Golgi, however, other phenotypes are not addressed here, and there are alternative explanations for this rather than a direct complementation. In addition to these issues, there are weak and incomplete phenotypes that are interpreted in a binary sense, there are incomplete experiments (switching between fibroblasts and KO cells). Finally, there are incomplete interpretations, for example- what exactly is causing the endosome swelling, what exactly is the nature of the GRASP interaction, what exactly is the GARP interaction doing? Overall I am supportive of the manuscript but I feel there are many small issues here that mean it is hard to draw a solid model of the new information at the end.

I have some more minor concerns below:

-For the VPS16B colocalisation imaging:

- 1) was the antibody validated
- 2) Some of the images (eg. the calnexin panel the VT11A panel) look different than the others.
- 3) I am surprised by the small error bars and clustered data compared the the variability looking at the images- how do the authors account for this?

-Did GRASP55 come up in the BioID?

- For VPS53 VPS16B localisation:

- 1) Did they colocalise with each other?
- 2) What about in the 16B KO cells?

- The authors write: "VPS53-DN localizes to the TGN, assembles into the GARP complex, and does not affect GARP-SNARE interactions". I am not convinced by this assertion as although indeed that is the mode of the DN mutant, of course all functions of the VPS53 endogenous will be affected. I suspect the authors understand this but maybe just worth clarifying.

- From the model at the end:

- 1) what is the evidence that the complex directly interacts with GARP?
- 2) what is the evidence that the complex directly interacts with GRASP55?
- 3) what is the evidence they colocalise as shown in the image?
- 4) how can they tell that the endosome phenotype is from complex-GARP defects rather than the dysfunctional golgi due to the GRASP55 interaction?

Reviewer #3

(Remarks to the Author)

I reviewed an interesting manuscript "The VPS33B-VPS16B protein complex facilitates Golgi ribbon formation and Golgi-endosome trafficking" which suggests a new insight into CHEVI tethering complex function as a complex involved in Golgi ribbon formation or maintenance.

The title "...facilitates Golgi ribbon formation and Golgi - endosome trafficking" is somewhat misleading. Are the authors suggesting all Golgi-endosomal trafficking is involved? Is it formation or maintenance of Golgi that is affected?

The authors call ARC syndrome "neonatal lethal" in several places. In actual fact these patients very rarely die in the neonatal period, they do not appear severely dysmorphic. The most severely affected patients die in the first 18 months of life due to specific platelet related bleeding defect or more commonly due to infections.

Previous publications suggested the defects in trafficking from Golgi to specific membrane bound vesicles or organelles, rather than a generalised dysfunction. For example in megakaryocytes only platelet alpha granule formation is defective but not other platelet granules. Similarly in the immune cells most of the membrane bound organelles are normally formed. In the renal cells very specific collagen modifying enzyme is abnormally trafficked.

The novel interactions of CHEVI complex are interesting and open new areas of research. The authors need to demonstrate how the abnormal Golgi morphology was controlled for the potential cell cycle changes in VPS33B KO cells. It was previously shown that CHEVI knockouts have increased cell proliferation. Furthermore, the VPS33B is a potential cancer suppressor gene. The knockout mice develop cancers.

Also interestingly Rab10 was previously reported to colocalise with CHEVI in its trafficking of PLOD3. RAB10 is not a canonical "Golgi matrix" protein, but it contributes to ribbon organisation via trafficking dynamics: continuous export helps prevent Golgi swelling and fragmentation.

Rab10-dependent trafficking supports membrane turnover and balance across cisternae.

Perturbation leads to Golgi compaction or fragmentation, mislocalisation of Golgi enzymes and cargo.

Furthermore, the authors suggest abnormal trafficking of cathepsin B to lysosomes due to a defect in retrograde M6PR trafficking. I gathered that the implication is that all lysosomal enzymes would be misdirected. However, this is not the findings in ARC patients. The lysosomal function is normal on testing in white blood cells. Thus there might be some other explanation for the provided experimental findings.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have improved the article significantly with text modifications and new data. Some key text edits include discussing the fact that the data is compatible with an association between VPS33B-VPS16B and GARP, or EARP or both protein complexes and the previously published VPS33B Bio-ID experiment (Hunter et al, 2018) that detected VPS51 and VPS53. Regarding new results, the additional investigation into Cathepsin D and anti-VPS16B antibody validation are quite helpful to strengthen conclusions. And new VPS16B localization data such as high values with Rab4 and Stx12 further suggest the possibility of roles at endosomes. A few minor presentation issues remain but they should be easily handled:

- The authors partially responded to the request of drawing the cell contours on fluorescence microscopy images, which are often not visible. To help the reader and to be consistent, please draw the cells on all figures showing fluorescence microscopy images.

- Figure 9a shows an alpha fold model containing two copies each of VPS16B and VPS33B, but the authors published data indicates the complex is composed of three copies of VPS16B and two copies of VPS33B. Unless there is new experimental evidence to support a tetramer rather than a pentamer, the current model may be misleading and it would be better to show a pentamer instead, even if the resolution of such structure is low.

- Proofreading could be more complete. Here are some examples:

Line 73: is "affinity" the best word here? Perhaps "relationship" or "analogous"

Line 91: missing word: ...has focused "on" a likely...

Line 164: missing word: ...similar to that seen "with" ARC...

Reviewer #2

(Remarks to the Author)

I thank the authors for their reply. They have carefully responded to my comments and performed some extra experiments. Overall, some of my original comments still stand. However, I consider it the authors' responsibility to decide whether to make the stated claims, and as they have acknowledged the relevant limitations in the manuscript, the paper has my support for publication.

Reviewer #3

(Remarks to the Author)

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Response to Reviewer comments

We thank the reviewers for their careful reading of the paper, and for valuable insights into matters requiring additional data and/or revisions of the text. We have made extensive revisions to clarify aspects including interpretations of observations and connections between our findings and those of other studies (e.g. regarding the CHEVI complex). Responses to specific comments are presented in blue text.

Reviewer #1 (Remarks to the Author):

This is a review of the manuscript titled “The VPS33B-VPS16B protein complex facilitates Golgi ribbon formation and Golgi-endosome trafficking” by Yao and co-workers. This work studies cellular defects in ARC syndrome, a severe disease caused by mutation of either VPS33B or VPS16B, proteins that associate into a tight complex. Significant previous knowledge on this topic has come from this research group. An important advancement presented here is the analysis of patient fibroblasts showing Golgi morphology defects both by electron and fluorescence microscopy (Fig. 1), extending previously recognized endo-lysosomal defects as well as deficiency in specialized organelles such as platelet granules. The study also provides nice new information on the endo-lysosome defects caused by ARC (for instance Fig. 7).

We thank the reviewer for appreciating the significance of our research.

However, other aspects of the manuscript appear somewhat preliminary as detailed below in the specific comments. Additionally, a previous VPS33B-Bio-ID screening detected VPS51 and VPS53 (Hunter et al, 2018, J. Mol. Biol.). This article needs to be appropriately cited, and the results compared with data presented here.

We are aware of the paper mentioned, which we cited in our previous JBC paper. We have also been in direct contact with the Gingras group regarding their BioID screen. A comparison of the results is presented in the revised Discussion, which raises some key points regarding our observations.

Moreover, as discussed by Hunter et al, VPS51 and VPS53 not only are components of the GARP complex, which mediates endosome-to-Golgi transport, but also the EARP complex, which mediates endosome-to-cell surface transport. The data is interpreted here as if only GARP could be a VPS33B-VPS16B partner when it could be either one of them or both, and the data presented here does not clearly distinguish between these possibilities.

This important point has been clarified throughout the revised paper, where VPS51 and VPS53 are described as components of GARP/EARP complexes, both of which potentially interact with VPS33B-VPS16B.

Major points:

1. Fig. 2: validation of the anti-VPS16B antibody needs to be shown. The Rab8 colocalization is significant and this Rab proteins is involved in transport to the cell surface. It would be important to use other markers of tubular endosomes like Rab4, STX12, retromer, or retriever. Comparison with GARP/EARP would be important also based on results presented later in the manuscript.

The anti-VPS16B Human Proteome Atlas antibody used here (HPA003589) has been extensively validated by the manufacturer for use in IHC and western blotting. Our validation for immunofluorescence is shown in new Supplementary Figure 2, which compares results for wild-type (WT) and hematopoietic-stem-cell specific *Vps33b* KO megakaryocytes, and WT and VPS16B KO HEK293 cells. We have also added data for colocalization of VPS16B with RAB4 and STX12, as well as VPS35 and VPS53, in new Supplementary Figure 4. These results are also mentioned in the revised Results, although we are wary of reading too much into them because unlike the endogenous protein pairs shown in Fig. 2b, it was necessary to use tagged/overexpressed constructs for VPS53 with VPS16B, and VPS16B with STX12 and RAB4,

owing to a lack of suitable antibody pairings for endogenous protein detection. A more extensive examination of these interactions will be the focus of future studies.

2. Fig 3. panels D and E: The staining pattern and localization of Myc-VPS16B is different from that of immunostained VPS16B (Fig. 2). Myc-VPS16B does not colocalize with the Golgi marker (Golgin-97). These data are not consistent with Fig. 2.

Figure 3 and its description/discussion have been revised to clarify our interpretation of these observations. The new Fig. 3d shows representative VPS16B-KO cells with/without VPS16B expression rescue by transfection with VPS16B-FLAG, quantified in Fig. 3e. The images indicate that VPS16B-FLAG distribution in rescue cells differs from that seen for endogenously-expressed VPS16B, consistent with what is often seen when tagged proteins are expressed at high levels relative to their endogenous counterparts. We believe that the Golgi morphology observations indicate that VPS16B-FLAG expression was sufficient to restore VPS33B-VPS16B function in Golgi formation.

3. Figure 4C: it would be important to perform co-IP of endogenous proteins, using the anti-VPS16B antibodies for the IP step and the VPS16B-KO HEK293 cells (or patient fibroblasts) as control. Additionally, in Fig. 4C (co-IP data with overexpressed proteins) it would help to have as an additional control a sample not expressing VPS33B-FLAG that is subjected to FLAG-IP to better control for GFP-GRASP55 non-specifically pulled down by FLAG-IP.

We agree that endogenous protein co-IPs for VPS16B and/or VPS33B would be useful. Unfortunately, here we face 2 problems: 1) lack of suitable antibodies (see response to comment 1); 2) both proteins are expressed at very low levels, and we have not been able to obtain reliable results with available antibodies beyond detecting endogenous VPS33B-VPS16B complex in prior studies. We have included negative controls for non-specificity of anti-FLAG conjugated beads used in this experiment in new Supplementary Figure 3.

4. Fig. 5A: as mentioned above, the same Bio-ID screening was previously reported by Hunter et al (2018, J. Mol. Biol). This publication and the fact that it also detected VPS51/53 should be mentioned and discussed. The Hunter paper used performed parallel VPS33A Bio-ID for comparison/ control. What control was used in the current study?

Untransfected HEK293 Flp-In cells, processed in parallel, were used as negative controls. As mentioned in the previous response, we have expanded discussion of the Hunter et al. findings to clarify the potential implications.

The possibility that VPS51/53 detected here are part of EARP complex needs to be incorporated or better yet experimentally test if it is GARP and not EARP (for instance by knocking down/out the specific subunit that distinguish each complex).

We have revised the text to reflect the possibility of involvement with GARP/EARP. We are content to leave the question of either/or interactions open for now, although it will be the subject of future studies. It remains possible that both interactions are associated with VPS33B-VPS16B function.

5. Fig 5B and C: Same comments as point #3 (co-IP of endogenous proteins, controls).

The challenges associated with endogenous protein co-IPs have been mentioned in the response to comment 3. We have described the controls for Fig. 5b,c in a new Supplementary Figure 3.

6. Fig 5I: Does Myc-VPS53 colocalize with VPS16B?

We have added colocalization observations and data for this pairing in new Supplementary Figure 4.

7. Line 261-264. While Magic red is a useful tool to study CatB, it really measures activity rather than the amount of the protein/enzyme per se. The activity of CatB can be affected by other factors like pH and the activity of other proteases that proteolytically activate it. To establish if there is less total catB, it would be important to analyze CatB by immunoblotting of cell extracts. This would also reveal if its processing is affected (different states, pre-protein, immature, mature can be identified by their size).

We appreciate this suggestion, and we have added new data and amended our original MR-CatB results in new Fig. 7. It was important to examine the maturation state of CatB, and as we now show, CatB processing is affected in both HEK293 VPS16B KO cells and ARC fibroblasts. We have added these observations to the results and Discussion.

8. Fig. 7F should be quantified.

Quantification of BafA treatment and related discussion have now been added to new Fig. 8 (previously Fig. 7) and the Results.

Minor points:

1. Affiliation # 7 for last author is not described

Affiliation #7 has been deleted.

2. Fig. 1C and many other figures showing cells: it is difficult to visualize the cell edge. Please indicate the cell contour for instance using a dotted line.

Cell contours have been indicated using dotted lines in the revised figures.

3. Line 159-160: "We conclude from these observations that VPS33B-VPS16B is not required for Golgi stack formation, instead likely plays a role in the formation and/or stability of Golgi ribbons." It is not clear how the authors derive this conclusion, at least at this point of the manuscript.

The manuscript has been revised by moving the Golgi stack formation comment to the section describing Fig. 1c, and by adding new Supplementary Figure 1 showing comparison of Golgi stacks in WT and ARC patient fibroblasts.

4. Line 186: Figure 4F should be called/indicated.

Figure 4f is now mentioned.

5. Lines 213-218. The meaning of this sentence needs to be clearer or perhaps move it to the discussion section.

This sentence has been removed.

6. Fig. 7F should be quantified.

Fig. 8F in the revised paper is now accompanied by Fig. 8i showing quantification of LAMP2+ vesicle diameters in cells without/with Bafilomycin A treatment. Interpretation of these results has also been added.

7. Fig. 7I, this quantification does not seem to match what is shown in Fig 7G. perhaps a more representative cell would help?

We appreciate the reviewer's suggestion and have replaced the previous image with a more representative cell to match the reported cresyl violet+ structure enlargement.

8. Line 285-287. "No clear effect of BafA treatment on vesicle morphology was detected in either normal or ARC fibroblasts, indicating that the abnormally large LAMP1+/RAB7+ vesicles present in ARC cells do not arise from V-ATPase dysregulation. Together, these observations reveal that

the formation of EELs is unlikely to be related to dysregulated ion transport, acidification or osmolarity". While the experiment is helpful, the conclusion seems overstated.

We agree that this could have been stated more accurately and have revised the discussion accordingly. While we may have ruled out V-ATPase, there are other possibilities awaiting investigation.

Reviewer #2 (Remarks to the Author):

Yao et al explore the function of the VPS33B-VPS16B complex. They use patient fibroblasts from ARC syndrome and observe that there is lower expression of both members of the complex, and the Golgi apparatus is structurally abnormal. They then localise VPS16B to the Golgi apparatus. They make and validate a VPS16B KO in which the phenotype appears similar to the ARC fibroblasts, and they complement the phenotype with overexpressed VPS16B. They show an interaction through co-IP with GRASP55 (a intergolgi tether) and VPS33B, and overexpression of GRASP55 seemed to recover the Golgi morphology. They then perform BIOID with VPS16B, and identify the GARP complex components, which they also show interact by co-IP. There was a minor decrease in the co-localisation of TGN46 and VPS53 in the APC cells. VPS16B was partially mislocalised in VPS53 DN cells.

CIMPR trafficking was assessed through localisation of the receptor which was mislocalised in the APC cells, and its ligand the lysosomal hydrolase was also less active. Finally, large endosomal like organelles were assessed as they were observed in TEM data, and they were shown to be properly acidified. Taken together, the authors present a model in which the VPS33B-VPS16B complex interacts with multiple Golgi machineries to modulate endosomal recycling and Golgi structure, which gives insights into disease pathology. The experiments are well performed and interpreted for the most part and the data clear and appropriately analysed.

We thank the reviewer for the positive feedback.

Despite these positive points, I have some concerns regarding the depth of mechanistic insight and the directness of all the findings. In short, the new interaction partners were validated by IP from cells, so may be indirect and there was no biochemistry or validation to demonstrate this. We agree with the reviewer that biochemical examination of protein interactions, such as via in vitro observations of recombinant purified proteins, can provide evidence of strong, direct and persistent molecular interactions, while co-immunoprecipitation (co-IP) from cellular lysates may be indicative of interactions that are sufficiently strong and persistent to be detectable, but they are not necessarily direct and may involve "unprobed" interactors. However, we consider that it is also important to acknowledge that Sec1/Munc18-like (SM) proteins and their associated complexes have been observed to participate in dynamic interactions with a wide range of proteins, complexes, membranes and vesicles. This potential complexity presents substantial challenges in resolving direct/persistent interactions, and while it is well worth pursuing in the future, we consider an extensive analysis of VPS33B-VPS16B interactions to be beyond the scope of the present study. What we believe we have shown here is that VPS33B-VPS16B associates with GARP/EARP and GRASP55 in cellular environments at physiological levels. Whether these interactions are direct or mediated by other components, they establish previously unreported functional aspects of Golgi ribbon maintenance and Golgi retrograde trafficking. We have revised the paper to mention that these interactions may involve a range of direct/indirect interactors.

Secondly, even if the interactions valid, the observations would be consistent with another model where VPS33B-VPS16B are doing something specific to the Golgi that indeed affects similar things to GARP and GRASP55. The best evidence of directness is the overexpression of GRASP55 on the complementation of the 16B Golgi, however, other phenotypes are not

addressed here, and there are alternative explanations for this rather than a direct complementation.

The reviewer raises a good point regarding the GRASP55 overexpression rescue. It is possible that overexpressing GRASP55 suppresses the VPS16B-deficient Golgi fragmentation phenotype through another mechanism such as direct tethering. The possibility that our observations are consistent with VPS33B-VPS16B and GARP/GRASP55 having co-operative and/or independent impacts on Golgi ribbon formation/integrity is now mentioned in the Results and Discussion.

In addition to these issues, there are weak and incomplete phenotypes that are interpreted in a binary sense, there are incomplete experiments (switching between fibroblasts and KO cells). Finally, there are incomplete interpretations, for example- what exactly is causing the endosome swelling, what exactly is the nature of the GRASP interaction, what exactly is the GARP interaction doing? Overall, I am supportive of the manuscript but I feel there are many small issues here that mean it is hard to draw a solid model of the new information at the end.

We agree that there are aspects of this study that fall short of ultimate elucidation of the biochemical/mechanistic aspects of the phenomena under investigation. We respectfully submit that this seems to set a rather high bar, and that achieving complete mechanistic elucidation would require data beyond the scope of this manuscript, and that this was not our goal here. This study began with an examination of primary fibroblasts derived from an ARC syndrome patient, wherein we identified cellular abnormalities that have not been previously examined in detail. These abnormal aspects included Golgi fragmentation and endosomal swelling, which likely cover a wide range of cellular processes where mechanistic aspects are under active investigation. We present evidence that VPS33B, VPS16B, and likely the VPS33B-VPS16B complex we have previously characterized, are involved in these processes, but clearly much more needs to be done to completely understand how. We have endeavoured to add clarity to our interpretations by extensively revising the Introduction, Results and Discussion. Alternative explanations have been taken into account, and we have endeavoured to maintain clear connections between observations, interpretations and conclusions.

I have some more minor concerns below:

-For the VPS16B colocalisation imaging:

1) was the antibody validated

We have presented the antibody validation for anti-VIPAS39 (Sigma HPA003589) in new Supplementary Figure 1. See also response to Reviewer 1, Major Point 1.

2) Some of the images (eg. the calnexin panel the VTI1A panel) look different than the others.

We tried to select cells representative of each VPS16B comparison set. We went back and included some that are more representative, including those in Fig. 2a showing calnexin and VTI1A co-staining. Importantly, the analyses shown in Fig. 2b and new Supplementary Figure 4e are based on data from many cells. It is also worth noting that, as we mention in the Results, VPS16B is expressed at low abundance, and Fig. 2a indicates that it is typically detected throughout the cell. At best, Fig. 2a provides a survey that helped guide subsequent examination of potential Golgi involvement by VPS33B-VPS16B.

3) I am surprised by the small error bars and clustered data compared the variability looking at the images- how do the authors account for this?

We have revised the graphs to eliminate unnecessary histogram bars that may have hindered interpretation. We have also clarified in the Methods that most bars indicate means and 95% confidence intervals. With regards to the apparent disagreement of images and numerical data, we can only say that the single images are meant to be representative, while the analysis is based on multiple images.

- Did GRASP55 come up in the BioID?

No, GRASP55 did not come up in our BioID screen. However, given the dynamic nature of cisternal stacking and ribbon formation, it seems likely that interactions involving GRASP55 and VPS33B-VPS16B may be transient and/or context-dependent, making them difficult to capture in vitro.

- For VPS53 VPS16B localisation:

1) Did they colocalise with each other?

2) What about in the 16B KO cells?

We have included data in new Supplementary Figure 4 regarding colocalization between Myc-VPS53 and VPS16B in WT HEK293 cells. Use of a tagged construct was necessary owing to lack of a valid VPS33B antibody suitable for immunofluorescence microscopy, to enable simultaneously detecting both endogenous proteins. While the data could be taken to indicate colocalization comparable to that observed for VPS16B and STX6, it is difficult to be confident about this. As we show in Fig. 3a, VPS16B KO cells do not express VPS16B.

- The authors write: "VPS53-DN localizes to the TGN, assembles into the GARP complex, and does not affect GARP-SNARE interactions". I am not convinced by this assertion as although indeed that is the mode of the DN mutant, of course all functions of the VPS53 endogenous will be affected. I suspect the authors understand this but maybe just worth clarifying.

We appreciate the opportunity to clarify this matter. VPS53 dominant negative was extensively characterized by Perez-Victoria and Bonifacino, as cited (*Mol Cell Biol.* 2009;29:5251-5263).

Their study investigated the multi-functional role of GARP and determined that VPS53 is required for the recycling of TGN46, additional to its function in GARP. We have revised the manuscript to better explain our rationale for the dominant negative experiment.

- From the model at the end:

1) what is the evidence that the complex directly interacts with GARP?

2) what is the evidence that the complex directly interacts with GRASP55?

3) what is the evidence they colocalise as shown in the image?

We now clarify that this figure presents a simplified schematic of proposed interactions involving relevant complexes/structures, possibly with other interactors involved. We have also included a structural model of VPS33B-VPS16B to aid visualization.

4) how can they tell that the endosome phenotype is from complex-GARP defects rather than the dysfunctional golgi due to the GRASP55 interaction?

We agree that could also be a possibility. Based on what is reported about GARP function and what we have observed, we propose that VPS33B-VPS16B deficiency could affect endosomes by inhibiting GARP/EARP function. There are undoubtedly other factors involved.

Reviewer #3 (Remarks to the Author):

I reviewed an interesting manuscript "The VPS33B-VPS16B protein complex facilitates Golgi ribbon formation and Golgi-endosome trafficking" which suggests a new insight into CHEVI tethering complex function as a complex involved in Golgi ribbon formation or maintenance.

We have revised the Introduction and Discussion in an effort to clarify the relationship of our findings to those reported for the CHEVI complex. As luck would have it, prior findings regarding CHEVI have been comprehensively reviewed in a hot of the press Nature Communications paper, to which we have directed interested readers. We have made it explicit that we interpret our findings here in the context of a stable VPS33B-VPS16B complex for which we determined a structure containing opposed VPS33B's (Liu et al. *J Biol Chem.* 2023; 299(6):104718) that may participate in multiple interactions. Since information regarding the precise composition and structure of CHEVI seems to be lacking, we chose the prudent path of admitting that we are

uncertain exactly how the CHEVI findings relate to those for VPS33B-VPS16B. They may be the same thing, but we just don't know at this point.

The title "...facilitates Golgi ribbon formation and Golgi - endosome trafficking" is somewhat misleading. Are the authors suggesting all Golgi-endosomal trafficking is involved? Is it formation or maintenance of Golgi that is affected?

Given the word limit for titles, we felt it necessary to be concise. The revised Results and Discussion clarify the specific aspects of these processes that were examined.

The authors call ARC syndrome "neonatal lethal" in several places. In actual fact these patients very rarely die in the neonatal period, they do not appear severely dysmorphic. The most severely affected patients die in the first 18 months of life due to specific platelet related bleeding defect or more commonly due to infections.

The reviewer is correct, consequently we have amended "neonatal" to "early". Like many such conditions, it is likely that many if not most cases fail in utero, as observed in mice where embryonic deletion of *Vps33b* is lethal (Dai et al. *J Pathol* 2016;240:108–119).

Previous publications suggested the defects in trafficking from Golgi to specific membrane bound vesicles or organelles, rather than a generalised dysfunction. For example in megakaryocytes only platelet alpha granule formation is defective but not other platelet granules. Similarly in the immune cells most of the membrane bound organelles are normally formed. In the renal cells very specific collagen modifying enzyme is abnormally trafficked.

The novel interactions of CHEVI complex are interesting and open new areas of research. The authors need to demonstrate how the abnormal Golgi morphology was controlled for the potential cell cycle changes in VPS33B KO cells. It was previously shown that CHEVI knockouts have increased cell proliferation. Furthermore, the VPS33B is a potential cancer suppressor gene. The knockout mice develop cancers.

We appreciate the understanding that Golgi morphology is connected to the cell cycle, and Golgi ribbons fragment during mitosis. To account for this, mitotic entry was inhibited before fixation to arrest all cells at interphase G0/G1, ensuring cells are compared at the same stage during the interphase. A clearer description of this control has been added to the Methods section of the revised manuscript.

Also interestingly Rab10 was previously reported to colocalise with CHEVI in its trafficking of PLOD3. RAB10 is not a canonical "Golgi matrix" protein, but it contributes to ribbon organisation via trafficking dynamics: continuous export helps prevent Golgi swelling and fragmentation. Rab10-dependent trafficking supports membrane turnover and balance across cisternae. Perturbation leads to Golgi compaction or fragmentation, mislocalisation of Golgi enzymes and cargo.

Furthermore, the authors suggest abnormal trafficking of cathepsin B to lysosomes due to a defect in retrograde M6PR trafficking. I gathered that the implication is that all lysosomal enzymes would be misdirected. However, this is not the findings in ARC patients. The lysosomal function is normal on testing in white blood cells. Thus there might be some other explanation for the provided experimental findings.

We have endeavoured to clarify our interpretations of observations in the revised Results and Discussion. As for the ARC observations, we chose to emphasize the aspects we could observe in primary cells, and concentrate on what we could examine using the HEK293 cell models. A comprehensive discussion of the diverse complexities of ARC syndrome symptoms, while undoubtedly of interest, is in our opinion beyond the scope this manuscript.

Responses to the Reviewers

Reviewer #1 (Remarks to the Author):

The authors have improved the article significantly with text modifications and new data. Some key text edits include discussing the fact that the data is compatible with an association between VPS33B-VPS16B and GARP, or EARP or both protein complexes and the previously published VPS33B Bio-ID experiment (Hunter et al, 2018) that detected VPS51 and VPS53. Regarding new results, the additional investigation into Cathepsin D and anti-VPS16B antibody validation are quite helpful to strengthen conclusions. And new VPS16B localization data such as high values with Rab4 and Stx12 further suggest the possibility of roles at endosomes. A few minor presentation issues remain but they should be easily handled:

- The authors partially responded to the request of drawing the cell contours on fluorescence microscopy images, which are often not visible. To help the reader and to be consistent, please draw the cells on all figures showing fluorescence microscopy images.

We appreciate the reviewer's request, and we have provided the cell contours on all the relevant fibroblasts and cells with outlines that are more difficult to see the perinuclear region. Some figures we chose to not contain the contours due to the difficulty of seeing the puncta themselves after adding the contours.

- Figure 9a shows an alpha fold model containing two copies each of VPS16B and VPS33B, but the authors published data indicates the complex is composed of three copies of VPS16B and two copies of VPS33B. Unless there is new experimental evidence to support a tetramer rather than a pentamer, the current model may be misleading and it would be better to show a pentamer instead, even if the resolution of such structure is low.

We have included a method section describing the model and why we present a tetramer rather than a pentamer.

- Proofreading could be more complete. Here are some examples:

Line 73: is "affinity" the best word here? Perhaps "relationship" or "analogous"

Line 91: missing word: ...has focused "on" a likely...

Line 164: missing word: ...similar to that seen "with" ARC...

We thank the reviewer for the edits. All of these mistakes have been amended.

Reviewer #2 (Remarks to the Author):

I thank the authors for their reply. They have carefully responded to my comments and performed some extra experiments. Overall, some of my original comments still stand. However, I consider it the authors' responsibility to decide whether to make the stated claims, and as they have acknowledged the relevant limitations in the manuscript, the paper has my support for publication.

We thank Reviewer 2 for the support.