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Dynamic Assembly of the Exomer Secretory Vesicle Cargo Adaptor Subunits

Martina Huranova, Gopinath Muruganandam, Matthias Weiss, and Anne Spang

Corresponding author: Anne Spang, University of Basel

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 11 July 2015

Thank you for the submission of your research manuscript to our editorial offices and your patience while we were conducting the peer review. We have now received the full set of reports on it.

As the reports are pasted below, I would prefer not to repeat the details of them here, but it becomes clear that while the reviewers agree in principle on the interest of the findings, they also all agree that in several instances, the data are over- or misinterpreted, which becomes clear mostly from the comments of referees 1 and 2. Nevertheless, in their reports the reviewers also suggest possible ways of addressing these issues, both experimentally and through textural changes and more careful interpretations. Upon further discussion with the referees in our cross-commenting process, the reviewers made additional suggestions, which I am paraphrasing here, as they are not part of the formal reports:

With regard to the issues raised by referees 1 and 2, the other reviewers agreed that additional experimental validation is necessary. The main criticism of this referee is a technical issue, namely the FCS calibration and controls (comment 1) and FRAP with fast moving objects (comment 2). Comments 3 and 4 refer to the addition of error bars and require caution in the interpretation, likewise in comments 5 and 6. In comment 7 referee 1 asks for an additional control.

To address comment 8, I would suggest testing the affinity by other techniques, for example with a competition assay. Likewise, to answer comment 10, I would recommend testing the association of Chs5 in the absence of ChAPs with membranes by subcellular fractionation and vice versa to find additional evidence for consecutive recruitment.

With regard to comments 2 and 3 of referee 3, it seems as if s/he would like to see additional independent data on ChAPs and Chs5 assembly and this should be addressed by a different complementary approach apart from microscopy techniques.

Given the potential interest of your findings, I would like to give you the opportunity to address the reviewers concerns and submit a revised manuscript with the understanding that all concerns of the referees must be fully addressed. Acceptance of the manuscript would require the full support of the referees in a second round of review.

I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. I do realize the amount of work required to address the concerns of the reviewers in full, but feel that without additional strengthening, referees 1 and 2 in particular are not in favor of publication of the study.

Should you decide to embark on such a revision, revised manuscripts should be submitted within roughly three months; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension can be obtained from the editor.

I look forward to seeing a revised form of your manuscript when it is ready. Should you choose to submit your paper elsewhere, I would welcome a message to this effect.

REFEREE REPORTS

Referee #1:

This study by Huranova et al. examines the in vivo dynamics of the exomer complex using quantitative live cell imaging. The authors examine how dynamics of each of the components changes in several different mutant strains. The result is an interesting and novel model for exomer assembly on the Golgi membrane. The experiments are very thorough and comprehensive, but I have several issues and questions about how the results are interpreted. In particular, there are claims about affinities, conformations, and the order of assembly that are not fully supported and I think alternative explanations are possible. Although there are many interesting observations presented in this manuscript, I cannot support publication of this manuscript in its current form.

Major points

1. Regarding the FCS data, the authors observe a fast component and a slow component for the exomer subunits. On Page 7, the authors state that the diffusion coefficient of the fast component is approximately equivalent to that expected from monomeric exomer proteins. Later on, on page 8 the authors state that the diffusion coefficient of the slow component corresponds to a particle the size of a vesicle (notably, it could also correspond to the size of a small TGN compartment). Yet on page 7 the authors are comparing differences in the % fast component versus % slow component for given subunits in wild-type and mutant cells, then seem to use these differences to draw the conclusion that "the majority of cytosolic ChAPs move independent of Chs5 presumably as monomers". However, the increase in the % of the fast component when chs5 is deleted should be interpreted to mean that less of the ChAP is on vesicles (or on the TGN). Furthermore, the value of the "fast" diffusion coefficients are increasing for 3 out of the 4 ChAPs in the chs5-delete cells (i.e., 16.5 for Bud7 in wt versus 18.2 for Bud7 in chs5-delete). This suggests that Bud7 is in a larger complex in wild-type cells (i.e, in an exomer tetramer), and then becomes monomeric when Chs5 is deleted. Considering that the diffusion coefficient was not calculated for free GFP (Table 1), I don't think the FCS experiments have been calibrated well enough to be able to state that the ChAPs are present predominantly as monomers in the cytoplasm based on the FCS data, and the authors seem to be ignoring the increase in the value of the fast diffusion coefficient (representing a decrease in particle size) when Chs5 is deleted. Based on these facts, I don't think the authors can rule out that exomer forms tetramers in the cytoplasm.

2. I am surprised that the authors are able to do FRAP experiments lasting ~60 seconds long on

yeast Golgi compartments, as these compartments move around in the cell in real time. Also, given the relatively short lifetime of Golgi compartments, how do the authors account for vesiculation events from the TGN? Presumably exomer complexes will disappear from the TGN when exomer vesicles are produced. It was not clear to me from the description in the Methods section how the authors dealt with these issues. The authors should add more description to the text of the Methods section to explain how they dealt with these issues.

3. Many interpretations of the FRAP data involve comparisons between residence times (1/k-off), yet the standard error (SEM) is not reported for these measurements, preventing the reader from assessing whether differences are significant. As the standard error is reported for the k-off values, it should be straightforward to report standard errors values for 1/k-off.

4. The authors state on page 10 "the residence time of Chs5 (8.2 s) in the absence of ChAPs was comparable to that of Arf1 (8.9 s, Fig. EV2B), suggesting that Chs5 binding to Arf1 is the initiating event of exomer assembly at the TGN." However, this result only tells you what happens when there are no ChAPs in the cell. It does not tell you anything about how the complex assembles on the membrane in wild-type cells (in the presence of ChAPs).

5. On the bottom of page 10 the authors state: "Moreover, these data also independently confirm that exomer assembly occurs on the TGN". I do not see how this statement is justified by the fact that the absence of different ChAPs changes the residence time of Chs5. An alternative explanation is that in the different mutant strains, different cargos are engaged by distinct pre-formed ChAP/Chs5 exomer complexes. Depending on the abundance of the corresponding cargos, this could result in the observed differences between Chs5 residence times.

6. On pages 12 and 16, the authors discuss differences between different ChAP/Chs5 exomer complexes and postulate that different ("closed" or "open") conformations observed in crystal structures of different ChAPs is the source of the observed differences in FRAP kinetics. However, other differences between the ChAPs are largely ignored, such as their different intrinsic affinities for lipid membranes and different cargos that they bind to. These differences are probably more important, and my guess is that the different conformation observed in the Chs6 crystal structure is a crystallization artifact - the crystal structure does not prove that this conformation predominates in cells, or that Chs6 prefers to adopt this conformation more so than does Bch1.

7. Along similar lines, the experiment presented at the top of page 13 is confusing. The authors transplant a PSSF "helix-breaking" sequence from Bch1 to Chs6 and then show that this does not have a major effect. However, judging from Figure S3A, Chs6 already has a PLLSL sequence in this region! Given that these experiments yield negative results, I suggest omitting them from the manuscript. Alternatively, perhaps a better way to do these experiments would be to swap the entire ~20 amino acid helix from Bch1 to Chs6 to see if there really is a difference.

8. On page 14, claims are made that Bch2 has the highest affinity for Chs5 and Bch1 has the lowest affinity for Chs5. What is the basis for these claims? Presumably the authors are deriving this from the FRAP data in the different mutant strains. However, this is not appropriate. The FRAP data provide you with apparent affinities for the TGN membrane environment, not for specific proteins. Chs5 and the ChAPs make multiple interactions at the TGN: with Arf1, with cargos, with membrane lipids, and with each other. It is not possible to use the FRAP data to make claims about just one of these interactions while ignoring the contributions of the others.

9. On page 16 in the Discussion, more claims about affinities of the ChAPs for Chs5 are made.

10. On page 17, the authors state "... first the scaffold is recruited and then two cargo adaptor proteins are selected". Given my stated concerns with the interpretations of the data, I do not think claims about the order of recruitment can be made. How do the authors know that Chs5 is definitely recruited before the ChAPs? Perhaps I am missing something that proves the order of events?

Minor points

11. For the experimental data summarized in Supplementary Table 1, why is the immobile fraction not calculated?

12. On page 16 in the Discussion, the others state that a previous study by another group found "overexpression of the N-terminal fragment of Chs5 was sufficient to expoert Chs3 to the plasma membrane (Paczkowski 2012)". Looking back at that paper, it appears that centromeric plasmids were used so it is not accurate to claim that the fragment was overexpressed.

13. I think the koff value reported for the Chs5-GFP in the Chs6 Bch2 delta delta strain (0.0109) may be a typo (off by a factor of ten).

Referee #2:

This paper focuses on exomer, a yeast-specific cargo adaptor that operates at the TGN to direct secretion of certain stress-responsive cargoes. The analysis provides evidence that unlike classical adaptors, exomer assembles onto the membrane in stages, with the scaffold protein Chs5 appearing first, followed by some subset of the four related cargo-binding ChAP subunits. Different ChAPs cooperate or compete with one another in a dynamic fashion to create a variety of adaptor complexes. Results shown here are interpreted in light of structural data to suggest that the preferred configuration in vivo is a pair of Chs5 molecules complexed to two different ChAP subunits in a 2:1:1 ratio. The specific configuration of the exomer complex seems to depend on expression levels as well as cargo interactions.

As I began reading this paper, I was favorably impressed by the use of quantitative microscopy to analyze the formation and dynamics of exomer complexes. The topic is interesting and the approach is potentially powerful. Thorough experiments yielded an abundance of quantitative data, and some of the basic conclusions are well supported by the data. In particular, it seems that the exomer complexes are dynamic and variable.

The problem was that as I progressed through the paper, I soon became lost in the sea of numbers and assumptions. The various perturbations have effects large or small, positive or negative, independent or cooperative or antagonistic. Interpretations build upon one another in a way that is not very persuasive. In the end, I honestly don't know what to make of the detailed results.

My suggestion is that the paper could be revised to focus on a few key points that are supported by straightforward experiments with clear-cut results. Then it would be possible to offer a constructive critique. As an example, here are some of the issues that came to mind as I read the first part of the paper.

1. In Fig. 1, please clarify how the TGN-to-cytoplasm ratio of fluorescence is measured. In any case, the claim that the Chs6-GFP and Bud7-GFP ratios are different at a significance level of $p<0.005$ is surprising given that the two bars are virtually identical. The authors should pay heed to the recent paper by L.G. Halsey et al. in Nature Methods.

2. Regarding Fig. 1, I am confused about the interpretation. On the one hand it seems that the ChAPs diffuse more slowly than GFP because they form complexes, but the detailed FCS analysis indicates that the majority of the ChAPs molecules are not in complexes at any given time. What am I missing?

3. Based on data suggesting that Chs5 and the ChAPs exist primarily as independently diffusing species in the cytoplasm, the interpretation is that Chs5 and the ChAPs come together to assemble the exomer complex on the membrane. But a nontrivial fraction (~25%) of the ChAPs molecules seem to be associated with Chs5 in the cytoplasm, so isn't it possible that these cytoplasmic complexes are the active form that assembles onto the membrane?

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The manuscript of Huranova et al. describes the individual contribution of exomer subunits to cargo selection and export. Using a combination of life-time measurement and photobleaching of functional GFP-tagged exomer subunits, the authors show that assembly of the exomer complexes

occurs in a sequential manner on membranes. Within this assembly pathway, the different ChAPs make different contributions to the stabilization on membranes. They use for this multiple ChAPs deletion strains and then monitor Chs5-GFP to determine the relative contributions to localization. For Bch1, overexpression is used to analyze the relative localization of cargoes such as Pin2 or Chs3. Their overall data are consistent with a model of dynamic preassembly of the complex, which then recruits cargo for carrier formation.

The authors present a overall well-conducted and very detailed study on the dynamics of the ChAPs relative to their main interaction partner Chs5. Their FCS and FRAP analyses with their different mutants suggest that each of the ChAPs make different contributions to the Chs5 stability and thus exomer formation, which likely depends on the available cargo.

My major concern with this study is the role of cargo in their analysis. I would consider it likely that the presence of cargo is rate-limiting for exomer-covered vesicles, while the exomer assembly might be dynamic as observed by the authors. It is exactly this point, where I find it important that they need to provide additional data to clarify the role of the Chs5-ChAPs crosstalk, potentially by using an additional or competing cargo and apply their techniques as nicely established now. Below is a list of my specific comments:

1) Figure 3B: The figure legend does not explain the red and green bars and the G to R, R to G reference. I understood that this refers to the Manders coefficient, though a better explanation in the legend is needed to clarify this also to the reader without flipping through the supplements.
2) Figure 4E: The authors suggest that Chs6 overexpression rescues the sorting of Chs3-GFP. I have difficulties following this by microscopy in their data as Chs3 is not found at the plasma membrane or the division site upon overproduction. I would also expect that such a mutant is calcofluor sensitive again, which may be a better way to demonstrate complementation.
3) The authors suggest in Figure 7 and 8 that cargo is required for efficient function. If I understood this right then overexpression of Bch1 reduces the available amount of Chs6 for Chs3-GFP export, which is a nice conformation for the authors' statement. Do these cells also become calcofluor resistant due to less Chs3 export? Can the effect of efficient Pin2 export, which seem to occur, be quantified? As for a comparative cargo with a clear substrate - does Chs6 overproduction also increase the amount of Chs5 localized to TGN membranes and possibly compete against the Bch1 levels?

Minor issues
1) Figure 2C: I do not see that Chs5 and Chs6 have almost the same expression level in the right panel. It might be a good idea to show a different blot that rather matches their quantification.
2) The authors mention that Chs5 is found on lipid droplets upon loss of other ChAPs. This might be a deposit also found for other proteins as recently discovered by Schuldiner and Kaganovich (Moldavski et al.), which could be mentioned.
3) I found the pictures rather small to appreciate the relative localization claimed by the authors in various figures. It might be a good idea to show enlargements of selected phenotypes such as the for me less obvious rescue in Figure 4E.

1st Revision - authors' response 13 October 2015

We would like to thank the reviewers for thoughtful comments and input. We believe that their comments helped us in preparing an improved manuscript.

Referee #1:

This study by Huranova et al. examines the in vivo dynamics of the exomer complex using quantitative live cell imaging. The authors examine how dynamics of each of the components changes in several different mutant strains. The result is an interesting and novel model for exomer assembly on the Golgi membrane. The experiments are very thorough and comprehensive, but I have several issues and questions about how the results are interpreted. In particular, there are claims about affinities, conformations, and the order of assembly that are not fully supported and I think alternative explanations are possible. Although there are many interesting observations

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Major points

1. Regarding the FCS data, the authors observe a fast component and a slow component for the exomer subunits. On Page 7, the authors state that the diffusion coefficient of the fast component is approximately equivalent to that expected from monomeric exomer proteins. Later on, on page 8 the authors state that the diffusion coefficient of the slow component corresponds to a particle the size of a vesicle (notably, it could also correspond to the size of a small TGN compartment). Yet on page 7 the authors are comparing differences in the % fast component versus % slow component for given subunits in wild-type and mutant cells, then seem to use these differences to draw the conclusion that "the majority of cytosolic ChAPs move independent of Chs5 presumably as monomers". However, the increase in the % of the fast component when chs5 is deleted should be interpreted to mean that less of the ChAP is on vesicles (or on the TGN). Furthermore, the value of the "fast" diffusion
coefficients are increasing for 3 out of the 4 ChAPs in the chs5-delete cells (i.e., 16.5 for Bud7 in wt versus 18.2 for Bud7 in chs5-delete). This suggests that Bud7 is in a larger complex in wild-type cells (i.e, in an exomer tetramer), and then becomes monomeric when Chs5 is deleted. Considering that the diffusion coefficient was not calculated for free GFP (Table 1), I don't think the FCS experiments have been calibrated well enough to be able to state that the ChAPs are present predominantly as monomers in the cytoplasm based on the FCS data, and the authors seem to be ignoring the increase in the value of the fast diffusion coefficient (representing a decrease in particle size) when Chs5 is deleted. Based on these facts, I don't think the authors can rule out that exomer forms tetramers in the cytoplasm.

As for the explanation of fast/slow pools: It seems that we have caused some misunderstanding here (see also next paragraph). We rephrased and clarified this part. We also included a description of the calibration for the FCS experiments in the Appendix Materials & Methods section.

2. I am surprised that the authors are able to do FRAP experiments lasting ~60 seconds long on yeast Golgi compartments, as these compartments move around in the cell in real time. Also, given the relatively short lifetime of Golgi compartments, how do the authors account for vesiculation events from the TGN? Presumably exomer complexes will disappear from the TGN when exomer vesicles are produced. It was not clear to me from the description in the Methods section how the authors dealt with these issues. The authors should add more description to the text of the Methods section to explain how they dealt with these issues.

All measurements were performed in cells in which the TGN was marked by Sec7-DsRed. This is now stated in the result part. We expanded the M&M section on the FRAP data acquisition.

3. Many interpretations of the FRAP data involve comparisons between residence times (1/k-off), yet the standard error (SEM) is not reported for these measurements, preventing the reader from assessing whether differences are significant. As the standard error is reported for the k-off values, it should be straightforward to report standard errors values for 1/k-off.

We incorporated the SEM into the Table 2.

4. The authors state on page 10 "the residence time of Chs5 (8.2 s) in the absence of ChAPs was comparable to that of Arf1 (8.9 s, Fig. EV2B), suggesting that Chs5 binding to Arf1 is the initiating event of exomer assembly at the TGN." However, this result only tells you what happens when there are no ChAPs in the cell. It does not tell you anything about how the complex assembles on the membrane in wild-type cells (in the presence of ChAPs).

We removed this statement.

5. On the bottom of page 10 the authors state: "Moreover, these data also independently confirm that exomer assembly occurs on the TGN". I do not see how this statement is justified by the fact that the absence of different ChAPs changes the residence time of Chs5. An alternative explanation is that in

the different mutant strains, different cargos are engaged by distinct pre-formed ChAP/Chs5 exomer complexes. Depending on the abundance of the corresponding cargos, this could result in the observed differences between Chs5 residence times.

The statement was removed.

6. On pages 12 and 16, the authors discuss differences between different ChAP/Chs5 exomer complexes and postulate that different ("closed" or "open") conformations observed in crystal structures of different ChAPs is the source of the observed differences in FRAP kinetics. However, other differences between the ChAPs are largely ignored, such as their different intrinsic affinities for lipid membranes and different cargos that they bind to. These differences are probably more important, and my guess is that the different conformation observed in the Chs6 crystal structure is a crystallization artifact - the crystal structure does not prove that this conformation predominates in cells, or that Chs6 prefers to adopt this conformation more so than does Bch1.

We completely removed this part from the manuscript.

7. Along similar lines, the experiment presented at the top of page 13 is confusing. The authors transplant a PSSF "helix-breaking" sequence from Bch1 to Chs6 and then show that this does not have a major effect. However, judging from Figure S3A, Chs6 already has a PLLSL sequence in this region! Given that these experiments yield negative results, I suggest omitting them from the manuscript. Alternatively, perhaps a better way to do these experiments would be to swap the entire ~20 amino acid helix from Bch1 to Chs6 to see if there really is a difference.

We followed the reviewer's advice and removed these data.

8. On page 14, claims are made that Bch2 has the highest affinity for Chs5 and Bch1 has the lowest affinity for Chs5. What is the basis for these claims? Presumably the authors are deriving this from the FRAP data in the different mutant strains. However, this is not appropriate. The FRAP data provide you with apparent affinities for the TGN membrane environment, not for specific proteins. Chs5 and the ChAPs make multiple interactions at the TGN: with Arf1, with cargos, with membrane lipids, and with each other. It is not possible to use the FRAP data to make claims about just one of these interactions while ignoring the contributions of the others.

We do agree with the reviewer that Arf1, lipids and cargo may contribute to the residence time of exomer components. Since we did not manipulate Arf1 and lipids in our setting, their contribution to the exomer recruitment should be the same for the different experiments. Moreover, we had shown previously that ChAPs recruitment to the TGN is mainly dependent on Chs5 (Trautwein et al. 2006). We have corroborated these findings by differential fractionation experiments, which are now part of the manuscript (Fig. 3A and C; EVFig 2B). All the data are in support of our interpretation about the apparent binding affinities.

9. On page 16 in the Discussion, more claims about affinities of the ChAPs for Chs5 are made.

We feel we are entitled to this discussion. We did change it, though to accommodate the reviewer's comment.

10. On page 17, the authors state "... first the scaffold is recruited and then two cargo adaptor proteins are selected". Given my stated concerns with the interpretations of the data, I do not think claims about the order of recruitment can be made. How do the authors know that Chs5 is definitely recruited before the ChAPs? Perhaps I am missing something that proves the order of events?

The model that we propose is based on the following:

1. from the FCS data we learnt that exomer is not preformed in the cytoplasm but assembles on the TGN
2. imaging data indicate that Chs5 is present on the TGN in the absence of the ChAPs but not vice versa (this manuscript and Trautwein et al., 2006; Pazckowski et al., 2012; Paczkowski and Fromme, 2014).

Thus the ChAPs depend on Chs5 for efficient recruitment and stabilization at the TGN, while Chs5

can bind independently, relying on the presence of activated Arf1 (Trautwein et al., 2006).
We agree that the model, which is put forward by the Fromme lab, is more consistent with an en bloc recruitment of exomer, but this is in contradiction with our FCS and FRAP data.

We performed the biochemical fractionation to demonstrate that the ChAPs are dependent on Chs5 for efficient TGN recruitment, but that the ChAPs are much less important for Chs5 recruitment (Fig. 3A and C; EVFig 2B).

Minor points

11. For the experimental data summarized in Supplementary Table 1, why is the immobile fraction not calculated?

The immobile fraction is now included.

12. On page 16 in the Discussion, the others state that a previous study by another group found "overexpression of the N-terminal fragment of Chs5 was sufficient to expoert Chs3 to the plasma membrane (Paczkowski 2012)". Looking back at that paper, it appears that centromeric plasmids were used so it is not accurate to claim that the fragment was overexpressed.

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Referee #2:

This paper focuses on exomer, a yeast-specific cargo adaptor that operates at the TGN to direct secretion of certain stress-responsive cargoes. The analysis provides evidence that unlike classical adaptors, exomer assembles onto the membrane in stages, with the scaffold protein Chs5 appearing first, followed by some subset of the four related cargo-binding ChAP subunits. Different ChAPs cooperate or compete with one another in a dynamic fashion to create a variety of adaptor complexes. Results shown here are interpreted in light of structural data to suggest that the preferred configuration in vivo is a pair of Chs5 molecules complexed to two different ChAP subunits in a 2:1:1 ratio. The specific configuration of the exomer complex seems to depend on expression levels as well as cargo interactions.

As I began reading this paper, I was favorably impressed by the use of quantitative microscopy to analyze the formation and dynamics of exomer complexes. The topic is interesting and the approach is potentially powerful. Thorough experiments yielded an abundance of quantitative data, and some of the basic conclusions are well supported by the data. In particular, it seems that the exomer complexes are dynamic and variable.

The problem was that as I progressed through the paper, I soon became lost in the sea of numbers and assumptions. The various perturbations have effects large or small, positive or negative, independent or cooperative or antagonistic. Interpretations build upon one another in a way that is not very persuasive. In the end, I honestly don't know what to make of the detailed results.

My suggestion is that the paper could be revised to focus on a few key points that are supported by straightforward experiments with clear-cut results. Then it would be possible to offer a constructive critique. As an example, here are some of the issues that came to mind as I read the first part of the paper.

Even though we would very much like to concentrate on one or two key findings in exomer assembly, the trouble is that the key point of the paper is about the contribution of all the different factors that we analyzed to exomer assembly and function. The idea is that exomer exports cargoes, whose localization is polarized and cell-cycle dependent and which are highly responsive to stress

(Zanolari et al., 2011; Ritz et al., 2014, as well as papers from the Schekman, Fromme and Roncero labs, as well as unpublished data on a novel exomer cargo from our group in collaboration with the Fromme lab). In addition, the polarized localization of the cargoes is strictly dependent on constant endocytosis and re-export from the TGN. Thus a dynamic equilibrium exists to ensure the proper steady state localization. Although all exomer cargoes respond to stress, they do this in a very distinct manner. For example, upon mild heat shock, Chs3 loses its polarized localization at the bud neck, and is now found all over the plasma membrane. The same stress has no appreciable effect on Pin2. In contrast, treating cells with LiCl causes Pin2 to be rapidly endcytosed and to be retained in the TGN as long as the stress persists. Exomer needs to be able to respond accordingly to these stresses and to export the right cargo at any given time. Of course the cargo will play a major role here. However the modularity of the exomer complex, the differences in abundance, affinities, the competition and collaborative behaviors all contribute to enable exomer to function properly. Each of the different parameters does not vary dramatically, however, this is exactly what one would expect for a fine-tuned complex that needs to be able to react to number of different situations. Hence except for data concerning the negative result on the helix interaction, we will go with the data we have and the requested additional experiments.
However, we acknowledge that the rational to perform this entire detailed analysis may not have been so clear and we re-wrote the manuscript to provide a clearer rational and made an effort to put the experiments better into context.

1. In Fig. 1, please clarify how the TGN-to-cytoplasm ratio of fluorescence is measured. In any case, the claim that the Chs6-GFP and Bud7-GFP ratios are different at a significance level of $p<0.005$ is surprising given that the two bars are virtually identical. The authors should pay heed to the recent paper by L.G. Halsey et al. in Nature Methods.

We respectfully disagree; the two bars are not virtually the same.

The reviewer questions the usefulness of p values, referring to a very recent paper. However we would like to point out that p values are still the accepted and widely used measure for statistical significance. In the absence of an alternative to display statistical significance, we will still use p values. Other readers may otherwise question whether we did some statistical testing at all.

2. Regarding Fig. 1, I am confused about the interpretation. On the one hand it seems that the ChAPs diffuse more slowly than GFP because they form complexes, but the detailed FCS analysis indicates that the majority of the ChAPs molecules are not in complexes at any given time. What am I missing?

We apologize for the confusion. We over-simplified the FCS data interpretation. What we can conclude from the FCS is that Chs5 and the ChAPs do not form a complex in the cytoplasm to any appreciable extent. Whether or not ChAPs form dimers or bind to other proteins in the cytoplasm, we cannot exclude. We re-wrote this part to make it clearer. See also the answer to comment 1 of reviewer 1.

3. Based on data suggesting that Chs5 and the ChAPs exist primarily as independently diffusing species in the cytoplasm, the interpretation is that Chs5 and the ChAPs come together to assemble the exomer complex on the membrane. But a nontrivial fraction (~25%) of the ChAPs molecules seem to be associated with Chs5 in the cytoplasm, so isn't it possible that these cytoplasmic complexes are the active form that assembles onto the membrane?

We can exclude that exomer assembles in the cytoplasm to any appreciable extent. The FCS data clearly show a very different diffusion behavior of Chs5 on one hand and the ChAPs on the other hand. We understand that the interpretation of the curves and the slower diffusing population is non-trivial. This is why we had included an example with just calculated curves in Fig. EV1C. We hope the modified paragraph makes thing clearer.

Referee #3:

The manuscript of Huranova et al. describes the individual contribution of exomer subunits to cargo selection and export. Using a combination of life-time measurement and photobleaching of functional GFP-tagged exomer subunits, the authors show that assembly of the exomer complexes

occurs in a sequential manner on membranes. Within this assembly pathway, the different ChAPs make different contributions to the stabilization on membranes. They use for this multiple ChAPs deletion strains and then monitor Chs5-GFP to determine the relative contributions to localization. For Bch1, overexpression is used to analyze the relative localization of cargoes such as Pin2 or Chs3. Their overall data are consistent with a model of dynamic preassembly of the complex, which then recruits cargo for carrier formation.

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My major concern with this study is the role of cargo in their analysis. I would consider it likely that the presence of cargo is rate-limiting for exomer-covered vesicles, while the exomer assembly might be dynamic as observed by the authors. It is exactly this point, where I find it important that they need to provide additional data to clarify the role of the Chs5-ChAPs crosstalk, potentially by using an additional or competing cargo and apply their techniques as nicely established now. Below is a list of my specific comments:

1) Figure 3B: The figure legend does not explain the red and green bars and the G to R, R to G reference. I understood that this refers to the Manders coefficient, though a better explanation in the legend is needed to clarify this also to the reader without flipping through the supplements.

Thank you for bringing this to our attention. A better explanation is provided.

2) Figure 4E: The authors suggest that Chs6 overexpression rescues the sorting of Chs3-GFP. I have difficulties following this by microscopy in their data as Chs3 is not found at the plasma membrane or the division site upon overproduction. I would also expect that such a mutant is calcofluor sensitive again, which may be a better way to demonstrate complementation.

We performed calcofluor drop assays and included them in Figure 4.

3) The authors suggest in Figure 7 and 8 that cargo is required for efficient function. If I understood this right then overexpression of Bch1 reduces the available amount of Chs6 for Chs3-GFP export, which is a nice conformation for the authors' statement. Do these cells also become calcofluor resistant due to less Chs3 export? Can the effect of efficient Pin2 export, which seem to occur, be quantified? As for a comparative cargo with a clear substrate - does Chs6 overproduction also increase the amount of Chs5 localized to TGN membranes and possibly compete against the Bch1 levels?

We performed calcofluor drop tests. To our surprise the Bch1 overexpression led to a hyper-sensitive growth behavior. Next, we stained chitin directly with calcofluor. Bch1 overexpression led to a strong reduction in plasma membrane chitin levels (Fig. 7D) consistent with the reduction of Chs3 plasma membrane localization. Chs3-independent changes to calcofluor sensitivity have been commonly observed when the downstream PKC and HOG signaling pathways were affected. The reduction in chitin at the plasma membrane after Bch1 overexpression is a strong argument for reduced Chs3 levels, independent of any signaling pathways.

To confirm that overexpression of Chs6 would reduce Bch1 and Chs5 TGN localization, we performed a FRAP analysis (Fig. 7 H and I). The overexpression reduced the immobile fraction in both cases, consistent with the live cell imaging results (Fig. 7F and G).

Minor issues

1) Figure 2C: I do not see that Chs5 and Chs6 have almost the same expression level in the right panel. It might be a good idea to show a different blot that rather matches their quantification.

We exchanged the panel.

2) The authors mention that Chs5 is found on lipid droplets upon loss of other ChAPs. This might be a deposit also found for other proteins as recently discovered by Schuldiner and Kaganovich (Moldavski et al.), which could be mentioned.

We only mention the lipid droplets in passing. In the revised version it is literally one sentence. We felt expanding this point would lend too much weight on this observation and may distract from the main message.

3) I found the pictures rather small to appreciate the relative localization claimed by the authors in various figures. It might be a good idea to show enlargements of selected phenotypes such as the for me less obvious rescue in Figure 4E.

We prefer to show more than just one cell to allow a better judgment and appreciation of the respective phenotypes. This comes at the cost that the cells are smaller. Where appropriate, enlargements have been included.

2nd Editorial Decision 25 November 2015

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, while both referees 1 and 3 now support publication in EMBO reports, referee 2 still has issues that in his/her opinion make the manuscript not well suited for publication in EMBO reports. On balance, and upon further discussions with the other referees, we would, however, be happy to offer publication of your study here once a few minor (and mostly formal issues) have been addressed, as listed below:

1. EMBO reports is using a number-based reference style and I would kindly ask you to change the current style using the attached end note file

2. I noticed that your manuscript contains Supplementary Methods. Please incorporate them in the main M&M section

3. For all figures and quantifications for which you have calculated error bars and/or standard deviations: Please indicate clearly how many independent times the experiment has been repeated (biological, not technical replicates). This information is currently missing for some of the data, e.g. in Fig 1 C and D.

4. Please provide us with a short, two-sentence summary of the manuscript and with two to three bullet points highlighting the key findings of your study

5. Please provide a schematic figure (in jpeg or tiff format with the exact width of 550 pixels and a height of about 400 pixels) that can be used as part of a visual synopsis on our website

6. Please fill out and send us a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision)

7. We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

Once you have made these minor revisions, please use the following link to submit your corrected manuscript:

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If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thanks again for your contribution to EMBO reports.

REFEREE REPORTS

Referee #1:

The revised manuscript is much improved, both in focus and in presentation and description of the results (in particular the FCS data). In my view the authors have adequately addressed the concerns of the reviewers and I am supportive of publication.

Referee #2:

My basic take on this paper has not changed. The authors have applied sophisticated techniques and have performed a large amount of careful, thoughtful work. Their ideas and interpretations are certainly interesting. But when it comes to the detailed experimental analysis, I simply cannot follow the arguments in many cases. The three issues that I cited as examples in my original review continue to puzzle me in the revision, and they were only the first things that confused me.

The other two reviewers seem to be more capable of following the logic of this manuscript. Therefore, in fairness to the authors, I suggest that those reviewers' recommendations should be heeded.

Referee #3:

The authors addressed my concerns. I have no further requests.

2nd Revision - authors' response 26 November 2015

I am very pleased that you provisionally accepted our manuscript.

I wish to thank you and the reviewers for helping us to improve the manuscript. In particular, I would like to thank reviewer 2, who albeit not being able to fully follow our arguments, was very fair.

We did the requested changes:

- we changed the reference style
- we incorporated all Material and Methods into the main manuscript, even though some methods
- were only used in the Extended Data section
- the numbers of cells analysed and the number of independent biological experiments are indicated
- throughout in the figure legends

In addition we provide a short summary, the take home message in bullet-points and as a schematic drawing.

We are in the process to assemble the source data, which will be submitted in due time

3rd Editorial Decision 27 November 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

