THE EMBO JOURNAL

Manuscript EMBO-2014-89032

TGF clusters COPII-coated transport carriers and promotes early secretory pathway organization

Adam Johnson, Nilakshee Bhattacharya, Michael Hanna, Janice Pennington, Amber L Schuh, Lei Wang, Marisa S Otegui, Scott M Stagg and Anjon Audhya

Corresponding author: Anjon Audhya, University of Wisconsin-Madison

Review timeline:

Submission date:	19 May 2014
Editorial Decision:	17 June 2014
Revision received:	13 November 2014
Editorial Decision:	24 November 2014
Revision received:	16 December 2014
Accepted:	17 December 2014

Editor: Andrea Leibfried

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 17 June 2014

Thank you for submitting your manuscript entitled 'A TFG-enriched meshwork facilitates COPII vesicle transport'. I have now received reports from all referees, which are enclosed below.

As you will see, the referees find your study interesting. However, they think that the current dataset does not sufficiently support your claims. They point out that additional data and information are needed and that alternative models on TFG functioning should be considered. Furthermore, technical concerns need to be addressed. Given the very constructive comments provided, I would like to invite you to submit a revised version of the manuscript, addressing all concerns of the referees. Please contact me in case of questions regarding the revision of your manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

The manuscript by Johnson et al., characterizes TFG protein structure and its role in organization of the early secretory pathway. Biochemical and structural (EM averaging) studies of human and worm TFG show that the N-terminal domain assembles into a cup-shaped octamer whereas the less structured C-terminal poly-Q rich domain can promote dimer formation and is required for

polymerization of purified TGF into 200-300 nm sized particles. High-resolution fluorescence imaging studies show that TFG aligns with ERES markers, COPII subunits and ERGIC markers in a spatial arrangement that could orient ERES toward the ERGIC. Indeed, knockdown of TFG or expression of mutant versions disrupts this organization, causes an increase in apparent COPII structures and uncouples ERES from the ERGIC. Based on the collective findings the authors conclude that TFG assembles into a meshwork that links ERES with the ERGIC and guides COPII-coated carriers to the ERGIC.

Overall these structural and cellular studies (8 main figures, 9 supplemental figures) are of high quality and support their proposed model. How ER-ERGIC organization is maintained and whether a macromolecular structure is needed at this interface has been a longstanding issue in the field, therefore these findings should be of general interest to the cell biology community. I have only a few minor concerns with the study as listed below.

1. Figure 2c and S1b are missing scale bars, which should be included.

2. Figure S8b/S8c is confusing because the text on page 15 comments that Sec16 is clustered around the periphery of enlarged TFG structures but figure labeling doesn't show Sec16 fluorescence under a condition where TFG is overexpressed? Please correct or clarify.

3. The conclusion that TFG assembles into an ERES-ERGIC meshwork is very appealing however the authors may want to be a bit more circumspect in this assertion since there is no direct evidence for such a structure in cells. For example in the first paragraph of the discussion the authors state that "Our data indicate that TFG functions in both capacities by forming a meshwork at the ER/ERGIC interface...." It might be better to state something like "Our data support a model in which TFG functions in both capacities by forming a meshwork...."

Referee #2:

This manuscript by Johnson and colleagues expands on previous work from the Audhya lab that identified TFG as a factor involved in ER export. The paper nicely demonstrates that TFG forms oligomeric structures that by EM analysis adopt a cup-like structure. Furthermore, these structures seem to assemble into higher-order structures that the authors propose act as a filter to prevent rampant diffusion of newly released COPII vesicles. The authors proceed to a thorough and high-resolution localization study that further characterizes different domains of TFG as important for various aspects of its assembly and examines the impact on COPII coat proteins and other secretory markers when TFG is abrogated. In all, the technical aspects of the work seem strong. Some minor additional experiments (below) would strengthen the conclusions. However, the major problem to my mind is that the
authors' model of TFG as a mesh-like trap for COPII vesicles, although appealing and exciting if true, is not fully supported. I'm not entirely convinced that the data do not point to alternative models for TFG function. This does not necessarily preclude the publication of the current work, but perhaps some of the more definitive statements might be toned down. For instance, the authors postulate the need for a mechanism to concentrate COPII vesicles prior to fusion. I'm not convinced of the need for such a mechanism to restrict diffusion of vesicles - these are very large structures and the cytoplasm is very dense. Another possibility for TFG function is of a more standard tethering factor that would promote homotypic fusion by interacting with the COPII coat, or as an assembly factor (which could also explain some of the data).

Specific comments:
1. Limited proteolysis of TFG under conditions +/- salt might support the model that the C-terminus is disordered in octomeric cup structure but drives assembly to larger ordered structures; this is also partially addressed by the GFP fusion experiments mentioned later in the ms (this observation could perhaps be mentioned earlier?).

2. Some antibody controls seem warranted since the TFG antibodies seem to be a novel reagent reported for the first time here: immunoblots of wt and tfg mutant lines seems important.

3. ERGIC-53 localization seems quite different in cells expressing mApple-Sec16B compared to

controls (Fig. S4) - is this consistently observed? It was hard to see if TFG localization might also be altered under these different conditions, given the few examples presented.

4. The claim for immunogold co-localization of TFG with areas of "highly enriched" COPII vesicles seems overstated when only 1 structure presented shows Sec31 labeling (Fig. 3e)

5. The claim for an oligomeric matrix of TFG that restrains COPII vesicles would be supported by FRAP/FLIP experiments, I guess TFG-GFP (at the C-term) might not be fully reliable, but Sec23-GFP might be illuminating.

6. Under conditions of TFG knockdown, are the scattered COPII puncta (Fig. 5) really vesicles or could they be soluble cytosolic pools of COPII coat proteins? Perhaps even aggregated into empty cages as a result of disrupted assembly? Are they on membranes?

7. The aggregation of COPII components into TFG-induced foci might also be indicative of a role for TFG in assembly rather than tethering/diffusion; if TFG modulates coat recruitment/polymerization, then one might expect a similar phenotype.

Referee #3:

In the current manuscript Johnson et al. characterized the structural role of TFG at the ER/ERGIC interface of COPII vesicular transport. The authors investigate the molecular properties of TFG using a broad set of experiments in vitro and in vivo. First, they observe and structurally characterize previously identified octamers of TFG using negative stain electron microscopy. The authors succeeded in a 3D reconstruction of cup-shaped octamers that is relatively low in resolution because of structural hetereogeneity. The molecular identity of the densities is left uninterpreteted. While the authors describe new properties of the C-terminal part of the protein, and they find their major role in contributing to polymerization and network-forming capabilities. Interestingly, they assign a COPII tethering role to the C-terminal PQ stretch of the protein. Based on these in vitro and other in vivo studies they arrive at a conclusion that TFG is forming a mesh-like network in the cell that prevents isotropic diffusion of COPII vesicles before they enter the secretory pathway.

While the authors provide a number of novel insights into the structure of TFG and puts forward a new hypothesis of the otherwise poorly understood function of TFG. Despite the importance of the manuscript, some of the conclusions are immature and require major revisions:
Major points:
1. The conclusion of the polymerization characterization is baffling. According to the authors, the C-terminal part is responsible for polymerization. This conclusion ignores the role of the PB1 domain that is known to polymerize in other proteins such as SQSTM1. As shown in the first part of the manuscript using EM characterization the N-terminal part of TFG has the ability to form the cup-shaped octamers and higher-order assemblies. It would be useful to characterize and show all used constructs by negative stain EM to arrive at a more balanced conclusion about the polymerization properties of TFG and compare whether higher-order structures in EM correlate with the determined particle size using confocal microscopy. Such a comparison would shed light on whether the particle size quantity is related to an ordered polymer formation or unspecifically aggregating.
2. Experimental conditions of polymerization are not well described (page 27: 'purified TFG isoforms ... were supplemented with a variety of salts at the concentrations indicated'). Details such as protein concentration and buffer composition and concentration should be mentioned in the text of the manuscript not only in the figure. Furthermore, it is not described which buffer conditions were used for the negatively stained EM samples.
3. The low-resolution negative stain EM part is presented inadequately. For example, the raw micrographs in Figure S1 and S2 do not contain scale bars. As the structure of octameric TFG is presented there are important structure determination statistics entirely missing from the manuscript. No resolution measure is given and no Fourier shell correlation is provided. I could not find the total number of particles that went into the presented density. It would be useful to know whether the authors have attempted to classify and remove particles or determined multiple structures because of the heterogeneity. Furthermore, I ask the authors to submit their EM density to the EM databank.
4. The authors state in the abstract that the TFG meshwork restricts isotropic diffusion. This very simplistic view of the cell in the abstract should be re-stated. I wonder whether there is anything in

the cell that isotropically diffuses apart from small compounds. I recommend a phrase instead that the authors themselves use in the manuscript: 'The TFG meshwork acts to locally concentrate COPII transport carriers'.
5. The authors state: "Moreover, the importance of this domain in TFG polymerization is consistent with prior findings, which indicate that disordered regions often exhibit a propensity to self-associate to form higher order polymers and fibrils under specific conditions (Frieden, 2007; Powers and Powers, 2006)." This statement including the references should be removed from the result section unless the authors provide evidence that this stretch has any propensity to form amyloid structures.

Some parts of the manuscript have statements or phrases that require minor editorial revisions:
1. page 5: 'high-resolution structural investigation' wording is not appropriate in the context of negative stain EM as it does not provide high-resolution structural data.
2. page 6: Typo: exhibited substantial flexibility
3. page 8: It is unclear which construct was used to measure structural disorder. Please add it to the text of the manuscript.

1st Revision - authors' response 13 November 2014

Response to Comments made by the Reviewers:

REFEREE #1

Reviewer #1: Overall these structural and cellular studies (8 main figures, 9 supplemental figures) are of high quality and support their proposed model. How ER-ERGIC organization is maintained and whether a macromolecular structure is needed at this interface has been a longstanding issue in the field, therefore these findings should be of general interest to the cell biology community. I have only a few minor concerns with the study as listed below.

We thank the reviewer for the kind comments regarding our manuscript.

Reviewer #1: Figure 2c and S1b are missing scale bars, which should be included.

We apologize for this oversight. We have confirmed that all images presented throughout the study now have scale bars.

Reviewer #1: Figure S8b/S8c is confusing because the text on page 15 comments that Sec16 is clustered around the periphery of enlarged TFG structures but figure labeling doesn't show Sec16 fluorescence under a condition where TFG is overexpressed? Please correct or clarify.

We apologize for the confusion here. We have revised the text to indicate that we examined the localizations of both Sec61b (Figure E8b) and Sec16B (Figure E8c) under conditions where TFG is overexpressed.

Reviewer #1: The conclusion that TFG assembles into an ERES-ERGIC meshwork is very appealing however the authors may want to be a bit more circumspect in this assertion since there is no direct evidence for such a structure in cells. For example in the first paragraph of the discussion the authors state that "Our data indicate that TFG functions in both capacities by forming a meshwork at the ER/ERGIC interface...." It might be better to state something like "Our data support a model in which TFG functions in both capacities by forming a meshwork...."

We entirely agree with the reviewer and have adjusted the text as suggested. Additionally, we made a point to tone down our assertion that TFG assembles into an ERES-ERGIC meshwork throughout the manuscript.

REFEREE #2

Reviewer #2: In all, the technical aspects of the work seem strong. Some minor additional experiments (below) would strengthen the conclusions. However, the major problem to my mind is that the authors' model of TFG as a mesh-like trap for COPII vesicles, although appealing and exciting if true, is not fully supported. I'm not entirely convinced that the data do not point to alternative models for TFG function. This does not necessarily preclude the publication of the current work, but perhaps some of the more definitive statements might be toned down. For instance, the authors postulate the need for a mechanism to concentrate COPII vesicles prior to fusion. I'm not convinced of the need for such a mechanism to restrict diffusion of vesicles - these are very large structures and the cytoplasm is very dense. Another possibility for TFG function is of a more standard tethering factor that would promote homotypic fusion by interacting with the COPII coat, or as an assembly factor (which could also explain some of the data).

We thank the reviewer for this criticism, and we have revised the manuscript significantly, paying close attention to tone down assertions that TFG acts as a mesh-like trap for COPII vesicles. We further consider additional models for TFG function. In particular, we now include data comparing the effects of inhibiting a known tethering factor that acts at the ER-ERGIC interface, the TRAPP complex, to TFG depletion (please see new Figures E9g, E9h, and E9i). Given the distinct phenotypes exhibited by cells treated with siRNAs targeting TRAPPC3 (mBet3) or TFG, it is unlikely that they act similarly to regulate the organization of the early secretory pathway. Although these data do not rule out a potential role for TFG as a tethering factor, they do distinguish its function from that of the TRAPP complex. We also carried out additional immunogold-EM experiments, which suggest that TFG depletion results in a loss of COPII vesicle clustering (please see new Figure E6f). Nonetheless, the reviewer's comment that a mechanism to concentrate COPII vesicles does not necessarily exist is well taken, and we have toned down statements suggesting this throughout the manuscript. To address a potential regulatory role for TFG in COPII coat assembly, we now include FRAP data, which show that GFP-Sec23A recovery in TFG depleted cells is similar to that in control cells (please see new Figure E6e and Movie E3). These data argue against a direct role for TFG in fostering the assembly of the COPII coat. Together, we believe the additional experiments included in the revision further strengthen the manuscript.

Reviewer #2: Limited proteolysis of TFG under conditions +/- salt might support the model that the C-terminus is disordered in octomeric cup structure but drives assembly to larger ordered structures; this is also partially addressed by the GFP fusion experiments mentioned later in the ms (this observation could perhaps be mentioned earlier?)

We thank the reviewer for this suggestion. We carried out the experiment proposed, and we found that TFG octamers are more sensitive to protease treatment when not assembled into larger polymers, consistent with the model that the carboxyl-terminus is disordered in the cup-like structure, but drives assembly into larger structures (please see new Figure E3c). With regard to the suggestion of reorganizing the flow of the manuscript, we felt that the GFP fusion experiments should follow the analysis of endogenous TFG localization. We therefore did not change the order of presentation.

Reviewer #2: Some antibody controls seem warranted since the TFG antibodies seem to be a novel reagent reported for the first time here: immunoblots of wt and tfg mutant lines seems important.

The manuscript includes a number of figures, which highlight the specificity of our TFG antibodies. In Figures 4A and 6A, immunofluorescence studies are used to demonstrate that depletion of TFG results in a lack of antibody staining in cells. Additionally, in Figure E6a, immunoblots are shown for control and TFG depleted cell extracts, indicating again that the TFG antibodies are specific. Finally, in a new Figure (E7e), we again highlight the specificity of the antibody by immunoblotting extracts from control cells and cells overexpressing TFG.

Reviewer #2: ERGIC-53 localization seems quite different in cells expressing mApple-Sec16B compared to controls (Fig. S4) - is this consistently observed? It was hard to see if TFG localization might also be altered under these different conditions, given the few examples presented.

We appreciate the reviewer's criticism. Based our on analysis of hundreds of images, we do not detect a significant difference in the localization of ERGIC-53 in control cells as compared to cells expressing mApple-Sec16B. However, the distribution of ERGIC-53 does look quite differently when comparing confocal images to those obtained by structured illumination microscopy (especially in the peri-nuclear Golgi region), which may have contributed to the reviewer raising this issue. Nonetheless, given the possibility that expression of mApple-Sec16B could affect the localization of other proteins in the early secretory pathway, we decided to replace a number of the figure panels throughout the manuscript with data using control cells (not expressing any transgenes) that were stained using antibodies that recognize endogenous proteins. The results using control cells as compared to cells expressing mApple-Sec16B did not differ.

Reviewer #2: The claim for immunogold co-localization of TFG with areas of "highly enriched" COPII vesicles seems overstated when only 1 structure presented shows Sec31 labeling (Fig. 3e).

We entirely agree with this criticism, and we have toned down the language used in the text. One possible reason for the scarcity of labelling is the fact that only a single, thin section is exposed to the antibody. Thus, while a number of vesicles appear in the EM image, relatively few molecules of Sec31A are exposed, which could interact with antibodies.

Reviewer #2: The claim for an oligomeric matrix of TFG that restrains COPII vesicles would be supported by FRAP/FLIP experiments, I guess TFG-GFP (at the C-term) might not be fully reliable, but Sec23-GFP might be illuminating. Under conditions of TFG knockdown, are the scattered COPII puncta (Fig. 5) really vesicles or could they be soluble cytosolic pools of COPII coat proteins? Perhaps even aggregated into empty cages as a result of disrupted assembly? Are they on membranes?

We thank the reviewer for suggesting this idea. We carried out a set of FRAP experiments, as mentioned earlier in this response. Importantly, these experiments further illustrated the dramatic increase in the number of COPII-labelled structures found throughout cells following TFG depletion. Rather surprisingly, our findings showed that GFP-Sec23A recovered with similar kinetics in the presence of absence of TFG. Interpretation of these data is not straightforward, however. We were restricted to the analysis of structures that remained in a single focal plane throughout the duration of the experiment (~20-30 seconds). Thus, the most highly dynamic structures, which move constantly in TFG depleted cells (please see Movie E2), could not be studied using this approach. Nonetheless, with this caveat in mind, our findings support the idea that the COPII-labelled structures found throughout TFG depleted cells are indeed transport carriers, since the recovery kinetics of GFP-Sec23A in cytosolic pools or aggregated empty cages would almost certainly be distinct from that of bona fide COPII vesicles.

Additionally, we have conducted new immunofluorescence experiments to show that the COPII-labelled structures that accumulate in TFG depleted cells are largely found juxtaposed to ER membranes (please see Figure E6d). Moreover, we show that a cargo (mannosidase II) accumulates in COPII-labelled structures, which are found independently of Sec16A in TFG depleted cells (please sees Figure 7B and 7C). Together, these data argue that the scattered COPII puncta are likely transport carriers.

Reviewer #2: The aggregation of COPII components into TFG-induced foci might also be indicative of a role for TFG in assembly rather than tethering/diffusion; if TFG modulates coat recruitment/polymerization, then one might expect a similar phenotype.

The reviewer makes a very good point. However, our FRAP studies suggest that COPII coats are recruited normally in the absence of TFG. Additionally, based on additional immunogold-EM studies (please see Figure E7g), we observe vesicular structures, which

exhibit a size consistent with them being COPII transport carriers, in and around the TFG-induced foci.

REFEREE #3

Reviewer #3: The conclusion of the polymerization characterization is baffling. According to the authors, the C-terminal part is responsible for polymerization. This conclusion ignores the role of the PB1 domain that is known to polymerize in other proteins such as SQSTM1. As shown in the first part of the manuscript using EM characterization the N-terminal part of TFG has the ability to form the cup-shaped octamers and higher-order assemblies. It would be useful to characterize and show all used constructs by negative stain EM to arrive at a more balanced conclusion about the polymerization properties of TFG and compare whether higher-order structures in EM correlate with the determined particle size using confocal microscopy. Such a comparison would shed light on whether the particle size quantity is related to an ordered polymer formation or unspecifically aggregating.

We apologize for any confusion. The reviewer is absolutely correct that PB1 domains exhibit the ability to homo-oligomerize. Our data indicate that the PB1 domain of TFG facilitates the formation of octameric cup-like structures, which are ~11 nm in diameter. However, the polymers we observe upon treatment with low concentrations of potassium acetate (300 mM) are significantly larger (~200-300 nm in size, as determined by negative staining EM and dynamic light scattering). Although it is feasible that the TFG PB1 domain may mediate associations between the 11 nm cup-like structures, we feel this is unlikely for multiple reasons. First, a truncated form of TFG lacking its carboxyl-terminal 100 amino acids exhibits a cytosolic distribution, even when overexpressed at high levels in cells. If the PB1 domain was sufficient to drive polymerization, we would have expected the truncated mutant to form large foci, as is observed for the full-length protein upon overexpression in cells. Second, when purified at high concentrations (~10 mg/mL), we fail to see the amino-terminal fragment of TFG form complexes that contain more than 8 subunits as determined by gel filtration coupled to multiangle light scattering, irrespective of salt additional *in vitro*. Instead, this domain appears to be largely limited to forming ~11 nm cup-like structures. Although we cannot rule out a role for the PB1 domain (or the coiled-coil motif) in promoting the polymerization of TFG into large 200-300 nm structures (as is now pointed out in the revised text), our data argue the PQ-rich carboxyl-terminal region plays an important role in this capacity.

All constructs described in the study that were amenable to recombinant purification were analysed by negative staining EM and shown in the manuscript. The size of polymers formed by the addition of ordering salts is ~200-300 nm, which equals the diffraction limit of the confocal microscope. Thus, it is challenging to precisely correlate particle sizes observed by EM and confocal microscopy. However, the particle size distributions measured by EM and dynamic light scattering are highly similar.

Reviewer #3: Experimental conditions of polymerization are not well described (page 27: 'purified TFG isoforms ... were supplemented with a variety of salts at the concentrations indicated'). Details such as protein concentration and buffer composition and concentration should be mentioned in the text of the manuscript not only in the figure. Furthermore, it is not described which buffer conditions were used for the negatively stained EM samples.

We apologize for these oversights. In the revised manuscript, we now describe the concentrations of proteins studied in the polymerization experiments, as well as the buffer composition and concentration. Similarly, we have amended the text to include a description of the buffer conditions and protein concentrations used for negative staining EM.

Reviewer #3: The low-resolution negative stain EM part is presented inadequately. For example, the raw micrographs in Figure S1 and S2 do not contain scale bars. As the structure of octameric TFG is presented there are important structure determination statistics entirely missing from the

manuscript. No resolution measure is given and no Fourier shell correlation is provided. I could not find the total number of particles that went into the presented density. It would be useful to know whether the authors have attempted to classify and remove particles or determined multiple structures because of the heterogeneity. Furthermore, I ask the authors to submit their EM density to the EM databank.

> We again apologize for these oversights. We have ensured that all images found in the figures contain scale bars. Additionally, we now provide a table, which details the structure determination statistics, including the resolution measurements and the Fourier shell correlations for all particles resolved using three-dimensional EM. These tables also include the total number of particles that were used in the densities. We have also added more details about how the data were processed and have submitted our EM densities to the EM databank. The details are as follows:
>
> EmDep deposition reference number 29696 has been assigned EMDataBank ACCESSION CODE **EMD-6075** for the map entry- **N-terminal C. elegans TFG**
>
> EmDep deposition reference number 29697 has been assigned EMDataBank ACCESSION CODE **EMD-6076** for the map entry- **N-terminal Human TFG**

Reviewer #3: The authors state in the abstract that the TFG meshwork restricts isotropic diffusion. This very simplistic view of the cell in the abstract should be re-stated. I wonder whether there is anything in the cell that isotropically diffuses apart from small compounds. I recommend a phrase instead that the authors themselves use in the manuscript: 'The TFG meshwork acts to locally concentrate COPII transport carriers'.

> We appreciate this criticism, and we have revised the text as suggested by the reviewer.

Reviewer #3: The authors state: "Moreover, the importance of this domain in TFG polymerization is consistent with prior findings, which indicate that disordered regions often exhibit a propensity to self-associate to form higher order polymers and fibrils under specific conditions (Frieden, 2007; Powers and Powers, 2006)." This statement including the references should be removed from the result section unless the authors provide evidence that this stretch has any propensity to form amyloid structures.

> We have removed this statement and the references from the manuscript, as suggested.

Reviewer #3: Some parts of the manuscript have statements or phrases that require minor editorial revisions:
1. page 5: 'high-resolution structural investigation' wording is not appropriate in the context of negative stain EM as it does not provide high-resolution structural data.
2. page 6: Typo: exhibited substantial flexibility
3. page 8: It is unclear which construct was used to measure structural disorder. Please add it to the text of the manuscript.

> We thank the reviewer for pointing out these issues. We have addressed all of them with appropriate text revisions.

2nd Editorial Decision 24 November 2014

Thank you for submitting the revised version of your manuscript for consideration by the EMBO Journal. It has now been seen by two of the original referees whose comments are enclosed. As you will see, both referees now support publication, pending minor changes. Referee #2 wonders whether TFG over-expression affects secretion. I don't know whether you have tested this, but you may want to discuss this point as outlined by referee #2. I would thus like to invite you to provide a final version of your manuscript.

A few editorial points need to be taken care of at this stage as well:

- please upload a modified manuscript text file; Please include also the number of cells analyzed for figures 4, 6, and 7 in the final version.

- please check whether all figure files are of adequate resolution and quality for production, and upload improved versions if necessary. In our routine check, we noted that the first confocal image panel in Figure 2B seems to be a dead panel/a black square. Please check this panel once more and replace if necessary.

- please suggest (in a cover letter) 2-5 one-sentence 'bullet points', containing brief factual statements that summarize key aspects of the paper - they will form the basis for an editor-drafted 'synopsis' accompanying the online version of the article. Please see the latest research articles on our website (emboj.embopress.org) for examples - I am happy to offer further guidance on this if necessary

- finally, it would also be great if you could provide a very basic model figure to be used for the synopsis. I think the final model figure of the paper could essentially be used as is, only it would have to be rearranged to fit best within the format restrictions of 550 pixels (width) x 150-400 pixels (height).

I am thus formally returning the manuscript to you for a final round of minor revision, to allow you to easily modify/replace the files. Once we have received them, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

REFEREE REPORTS

Referee #2:

The authors have addressed my previous concerns by the inclusion of new data and more circumspect language in the manuscript. My one remaining question is whether the COPII-positive foci induced upon TFG over-expression cause a delay in secretion. I know the authors report no changes in Golgi morphology, but one would imagine that secretion specifically would be impacted if this is a sink for COPII vesicles. Some mention should be made of this possibility even if the authors have not yet explicitly looked at acute secretion events.

Referee #3:

I am happy with all revisions of the manuscript. The authors have sufficiently addressed my raised concerns. In particular, the technical inconsistencies of the EM data processing have been improved as the authors added the FSC statistics and EM databank deposition IDs. Therefore, the manuscript can be published in its current form.

2nd Revision - authors' response 16 December 2014

We are pleased that the reviewers now support publication. Over the course of the past few weeks, we have made several attempts to measure the kinetics of protein secretion following TFG overexpression (both using transient transfection and retroviral-mediated infection). Unfortunately, we were unable to make a definitive conclusion, since the expression of commonly used cargo molecules (such as VSVG-GFP) induce ER stress and rapidly promote apoptosis when TFG is also overexpressed. Nevertheless, we now include a discussion of this point in the text, as suggested by reviewer #2. The modified manuscript also includes the number of cells analyzed for all studies (detailed in the figure legends), and we have ensured that all figure panels are of adequate resolution. In particular, we have replaced one of the panels in Figure 2 as suggested.

We also developed a series of bullet points, as requested:

- TFG monomers self-associate to form flexible, octameric cup-like structures *in vitro*.

- TFG complexes polymerize at the ER/ERGIC interface to concentrate COPII-coated transport carriers.

- Depletion of TFG disrupts the ER/ERGIC interface and impairs COPII-mediated protein trafficking.

We have also modified Figure 8 to fit within the format restrictions for use in the synopsis. Once again, thank you for your consideration, and I look forward to hearing your decision regarding publication of our manuscript.

