Sec71 separates Golgi stacks in *Drosophila* S2 cells

Syara Fujii, Kazuo Kurokawa, Tatsuya Tago, Ryota Inaba, Arata Takiguchi, Akihiko Nakano, Takunori Satoh and Akiko K. Satoh
DOI: 10.1242/jcs.245571

Editor: David Glover

Review timeline
Original submission: 18 May 2020
Editorial decision: 13 July 2020
First revision received: 12 October 2020
Accepted: 18 November 2020

Original submission

First decision letter

MS ID#: JOCES/2020/245571

MS TITLE: Sec71 separates Golgi stacks in Drosophila S2 cells

AUTHORS: Syara Fujii, Kazuo Kurokawa, Tatsuya Tago, Ryota Inaba, Arata Takiguchi, Akihiko Nakano, Takunori Satoh, and Akiko Kono Satoh
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

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

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

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

Reviewer 1

Advance summary and potential significance to field

In this paper, Fujii and colleagues describes the aggregation of Golgi-stacks upon BFA treatment, the formation of so called BFA-bodies. The major novelty of this work is finding a mechanistic insight in formation of Golgi aggregates and revealing the importance of Sec71 guanine-nucleotide exchange factor in this process. Using appropriate genetics and cell biology techniques they demonstrated that Sec71 is the sole BFA target in Drosophila and is involved in Golgi stack separation. Besides using cultured cells, the authors also characterized Sec71 function in photoreceptors of intact flies. In addition to the main line of the story, providing data for two additional biological questions increases the quality of the work (and are perfectly fitted in the main line of thoughts): we get bit insight into the dynamics of merging and separation of Golgi stacks and learn about another ARF-GEF, Garz which turned out to be not a BFA target but is essential for the maintenance of Golgi stacks.
The results described are significant for the Golgi field because it broadens the understanding Golgi organization (both in Drosophila and in general), dynamics and describes two Drosophila genes influencing this process. In addition, it gives mechanistic insight into BFA effect in Golgi aggregation which can have methodological significance for future experiments.

Although the data is generally well supporting all the conclusions, there were some critical inconsistencies in the use of Sec71 mutant variants. Another problem is that at some key experiments the quantification is missing. Since the penetrance of BFA-body formation is relatively low even at high BFA concentration or in Sec71 mutants (around 20% of the cells form BFA-bodies, the majority of the cells do not form these structures), the quantification and indication of the percentage of cells giving the phenotype are essential for the evaluation and comparison of different Sec71 mutant variants. Alongside with other minor issues, IÂ'm going to indicate and comment on these issues bellow. I think it is essential to address these points before accepting this manuscript for publication.

Being aware of the uncertainty and possible difficulties to do lab experiments in the time of COVID-19 pandemic, I believe that reviewing the major issues of this manuscript is entirely possible based on data and raw microscopic images already available in the hands of the authors.

Comments for the author

Major remarks

Remark 1 - Page 5 : "Less than 20% of S2 cells showed aggregation of Golgi stacks, TGN, and RE."

Please specify in the text that "less than 20% of BFA-treated S2 cell showed ...". From the context, it is obvious that it refers to the effect of BFA, but semantically it means that S2 always (even the wt) show aggregation. It is important to show (at least in the legends) that how many cells were counted (n=?), how many times the experiment (BFA treatment) was repeated. Only based on this data can a reviewer/reader asses the repeatability/consistency of the appearance of BFA-bodies.

Remark 2 - Page 5: "Both mTq2::Rab11 and Rab6 localized to the central domain of the aggregate, but there was a clear difference in the localizations: mTq2::Rab11 localized only to the central core, but Rab6 localized more broadly."

Please, make a one-two sentence comment on this difference either here in the results section or in the discussion that what is the (possible) explanation of this difference.

Remark 3 - Page 7: "These results indicate that Sec71is the target of BFA, and the impairment of Sec71 activity results in the formation of BFA-bodies."

The authors cannot make this statement at this point of the results section (you can do this later, see Remark 5). Similarity between the BFA induced and the Sec71 dominant-negative induced Golgi aggregates is not a poof for Sec71 being target of BFA. You need to present first the direct link between BFA and Sec71. You do this in the next results section, so do this statement there.

Please, replace it here with something like "Sec71 loss-of-function phenocopies the BFA-treatment and results in structures similar to BFA-body formation".

Remark 4 - Page 7: “In contrast, S2 cells with Sec71 F713Y responded to a low concentration of BFA (5 μM)”

Please, answer here if the F713Y BFA-bodies have the same or similar structure compared to BFA-bodies characterized previously in WT cells. Were the size and density and even the marker distribution of these BFA bodies similar? This careful examination would be particularly important because there seems to be a difference in marker distribution between BFA bodies pointed with arrows on figure 2 L (right panel) and 2 N (left panel). Since authors use this line to investigate the mechanism of BFA body formation later, it is particularly important to clarify the similarities/differences here.

Remark 5 - Page 8: “These results indicate that the impairment of Sec71 is necessary and sufficient for BFA-body formation. Thus, Sec71 is the only BFA-target responsible for BFA-body formation.”

This concluding sentence is correct here and this is the place of that statement you made earlier (see Remark 3).

Remark 6 - Page 8: “In S2 cells with Sec71 M717L incubated with 25 μM BFA, Sec71 was concentrated at the center of BFA-bodies, accompanied by Rab11, however, Golgin245 was mostly found between GM130 and Sec71 (Fig. 3A, B lower panel and plots).”

First of all, there is and inconsistency between the labeling of the Figure 3, the figure legends and this sentence in results section. On Figure 3 itself there is no indication that we are looking at M717L mutant cells. According to the figure legends, cells on Fig3A are M717L mutant and cells on Fig3B are wild type S2. This sentence in the Results section says that both are M717L mutants.

It is not understandable why the authors used the M717L variant to demonstrate the localization of Sec71. They described in the preceding paragraph that M717L variant is resistant for BFA body formation (only 1% of cells show BFA-bodies after 50uM BFA treatment), but inconsistently with this, without any explanation, they used the very same resistant variant to show the localization of Sec71 (in this case Sec71M717L mutant) in BFA-bodies after treating the cells with 25uM (!) BFA. There are no statistics presented here. What was the percentage of M717L cells showing BFA-bodies with 25 uM BFA? If the formation of these structures is dependent on the dose of BFA, one can expect even less than 1% BFA-body formation in this case. Do the authors think or have evidence that the small number of BFA-bodies formed in M717L BFA resistant cells are accurately representing the structure of BFA-bodies in wild type cells and the accurate localization of Sec71?

Since they used lower BFA concentration here, this raises the question of whether the penetrance BFA-body formation is dose-dependent or not. Do the authors have data or are there data available in the literature that lower BFA concentration results is fewer BFA-bodies. It is not clear if 50 uM BFA is a plateau concentration above which you cannot see more BFA-body formation or it is a sort of balanced concentration where you can observe BFA-body formation but is not very toxic for the cells. Please, explain these points.

Remark 7 – Page 8: “As the population of the wild type S2 cells responding to high concentration of BFA was limited, we used BFA-hypersensitive Sec71 F713Y S2 cells...”

More or less the same issue as Remark 6. Because of the lack of quantification, we don't know the frequency of BFA-body formation at this condition. We do know that F713Y responds to 5uM BFA to an extent as WT cells respond to 50uM BFA (i.e. roughly 20% of the cells show BFA body formation). How is the F713Y react to 25uM BFA? Please indicate if this increase in BFA concentration increases the frequency of BFA body formation (compared to 5 uM BFA used before). Is the structure of BFA bodies different by structure upon higher BFA concentration (i.e. BFA body size or density or even marker distribution change ?). I think it is crucial to clarify these details to justify the use of F713Y with 25uM BFA instead of WT with 50uM BFA.

Remark 8 – Page 8: “The merger and separation of TGNs was constantly repeated in BFA-untreated cells”
In order to more convincingly demonstrate the merging events of Golgi stacks, it would be great if authors could shoe a side view of Z-stacks of some images where the merging happens thus demonstrating that real merging/fusion event happened and not just superposition of two stacks in different depth which from a top view looks like merging.

Minor remark:

Discussion, page13, “...TGN/RE is central in BFA-bodies surrounded by Golgi stacks on the trans-side”

here I would suggest using the word “periphery” instead of “trans-side” to improve the clarity since “trans-side” in this context can have several different meanings.

Discussion, page 13, “...at low BFA concentration in high-penetration (Fig. 2J-M).”
Maybe an typo here. To be precise, you should refer to Fig. 2J-O.

Reviewer 2

Advance summary and potential significance to field

Brefeldin A (BFA) has been used for decades as one of the most specific small molecule blockers of the secretory pathway. It inhibits the GEFs that activate the Arf GTPases on the Golgi. However, there are two main classes of Arf GEF and although BFA acts on both in many mammalian cells, in other species it acts on only one or the other. As a result, the structure of the Golgi following BFA treatment varies depending on the species. This paper reports a careful study of the effect of BFA in Drosophila cells. It had previously been shown that BFA blocks secretion, but the mechanism was unclear. This paper reports a series of well-designed and carefully quantified experiments that show clearly that in Drosophila S2 cultured cells, BFA acts solely on the Sec71 GEF of the trans Golgi network (TGN) and on no other cellular target. The authors go on to show that when S2 cells are treated with BFA then in a subset of cells the Golgi stacks coalesce to form a Â“BFA bodyÂ” in which a central conglomeration of TGN and recycling endosome markers is surrounded by discrete elements containing early Golgi markers. The authors argue that this means that Arf activation is required for the TGN to separate from the rest of the stack. They also express a dominant negative version of Sec71 in photoreceptor cells and find that structures like BFA bodies appear with a concomitant block in delivery of proteins to the cell surface.

Overall this is a very high quality study which clearly demonstrates how BFA acts in Drosophila, whilst also providing new insight into the role of Arf in vivo. As such it is potentially suitable for publication, but there are a few minor technical issues that I feel need to be addressed, and also the authorsÂ’ interpretation of the mechanism behind Golgi body formation needs to be more clearly explained and justified. These points are outlined in the suggestions to authors.

Comments for the author

a) The authors should explain why Golgi stacks aggregate in only 20% of the treated cells.

b) The authors localise Rab11 using a fusion to Tq2. Have they tried using an antibody to look at the endogenous Rab11?

c) Is it possible that the TGN and the Golgi-associated recycling endosome are the same thing? For instance Sec71-V5 looks very similar in distribution to Rab11. Also, do AP-1 and Rab11 co-localise?

d) The authors refer to the localisation of GalT::mTq2 on page 6, but do not mention which figure.

e) The authors state that BFA-bodies form in 22.6% of Sec71F713Y cells with 5 micromolar BFA. What is the frequency of BFA bodies in such cells treated with the 50 micromolar levels used elsewhere in the paper, or the 25 micromolar used with this particular mutant in Figure 4?

f) One key point is that I was confused by what the authors meant by “separation” when referring to Golgi stacks or TGNs. Do they mean the TGN separating away from the rest of the stack or do they mean the TGNs on two different stacks merging together to make one and then splitting apart to make two – ie scission? It is not clear to me as either seem possible (or even both at the same time). The authors also refer to “TGN/RE” separations which added to my confusion. It would help if the authors have a summary model figure in the Discussion to illustrate what they think is happening.

g) The movie showing BFA body formation suggests that the stacks move toward each other to form the structure. Does the BFA body form next to the centrosome? Is formation blocked by depolymerising microtubules?

h) MPPE also seems to be present in the rhabdomeres as well as the Golgi (Figure 6). If this is correct the authors should note this and not simply state that MPPE is Golgi marker.

i) There are also a few minor typos (eg marge instead of merge, and “action mechanism” instead of “mechanism of action”), and Drosophila is sometimes not in italics.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

In this paper, Fujii and colleagues describes the aggregation of Golgi-stacks upon BFA treatment, the formation of so called BFA-bodies. The major novelty of this work is finding a mechanistic insight in formation of Golgi aggregates and revealing the importance of Sec71 guanine-nucleotide exchange factor in this process. Using appropriate genetics and cell biology techniques they demonstrated that Sec71 is the sole BFA target in Drosophila and is involved in Golgi stack separation. Besides using cultured cells, the authors also characterized Sec71 function in photoreceptors of intact flies. In addition to the main line of the story, providing data for two additional biological questions increases the quality of the work (and are perfectly fitted in the main line of thoughts): we get bit insight into the dynamics of merging and separation of Golgi stacks and learn about another ARF-GEF, Garz which turned out to be not a BFA target but is essential for the maintenance of Golgi stacks.
The results described are significant for the Golgi field because it broadens the understanding Golgi organization (both in Drosophila and in general), dynamics and describes two Drosophila genes influencing this process. In addition, it gives mechanistic insight into BFA effect in Golgi aggregation which can have methodological significance for future experiments.

Although the data is generally well supporting all the conclusions, there were some critical inconsistencies in the use of Sec71 mutant variants. Another problem is that at some key experiments the quantification is missing. Since the penetrance of BFA-body formation is relatively low even at high BFA concentration or in Sec71 mutants (around 20% of the cells form BFA-bodies, the majority of the cells do not form these structures), the quantification and indication of the percentage of cells giving the phenotype are essential for the evaluation and comparison of different Sec71 mutant variants. Alongside with other minor issues, I’m going to indicate and comment on these issues bellow. I think it is essential to address these points before accepting this manuscript for publication.

Being aware of the uncertainty and possible difficulties to do lab experiments in the time of COVID-19 pandemic, I believe that reviewing the major issues of this manuscript is entirely possible based on data and raw microscopic images already available in the hands of the authors.

Reviewer 1 Comments for the Author:

Major remarks

Remark 1 - Page 5 : “Less than 20% of S2 cells showed aggregation of Golgi stacks, TGN, and RE.”

Please specify in the text that “less than 20% of BFA-treated S2 cell showed ...”. From the context, it is

obvious that it refers to the effect of BFA, but semantically it means that S2 always (even the wt) show aggregation.

Thank you so much. We specified as you suggested. In our new quantification, about 30% of BFA-treated S2 cells showed BFA-bodies. Thus, we changed to "About 30% of BFA-treated S2 cell showed ...".

It is important to show (at least in the legends) that how many cells were counted (n=?), how many times the experiment (BFA treatment) was repeated. Only based on this data can a reviewer/reader asses the repeatability/consistency of the appearance of BFA-bodies.

In this original experiment for Figure 2L-O, we counted more than 150 cells for both BFA-treated and not treated for in WT and mutant cells in each experiment. We did this experiment three times.

We now investigated the dose-dependence of WT, F713Y, M717L for BFA and Figure 2L-O were replaced by new data sets. In this new experiment, we counted more than 250 cells for both BFA-treated and not treated WT and mutant cells at each concentration of BFA. We repeated this experiment three times. We modified the text in Fig.2 legend accordingly.

Remark 2 - Page 5: "Both mTq2::Rab11 and Rab6 localized to the central domain of the aggregate, but there was a clear difference in the localizations: mTq2::Rab11 localized only to the central core, but Rab6 localized more broadly." Please, make a one-two sentence comment on this difference either here in the results section or in the discussion that what is the (possible) explanation of this difference.

We added the following sentences after the sentence you cited.
"In untreated cells, Rab11 localizes specifically on the RE, whereas Rab6 locates broadly from trans-Golgi to the RE. The arrangement of GalT, Rab6 and Rab11 in the BFA-induced aggregate likely reflects that in normal cells."

Remark 3 - Page 7: "These results indicate that Sec71 is the target of BFA, and the impairment of Sec71 activity results in the formation of BFA-bodies."
The authors cannot make this statement at this point of the results section (you can do this later, see Remark 5). Similarity between the BFA induced and the Sec71 dominant-negative induced Golgi aggregates is not a poof for Sec71 being target of BFA. You need to present first the direct link between BFA and Sec71. You do this in the next results section, so do this statement there.
Please, replace it here with something like "Sec71 loss-of-function phenocopies the BFA-treatment and results in structures similar to BFA-body formation".

I agreed your indication and thank you so much for providing the good sentence. We changed the sentence as you suggested.

Remark 4 - Page 7: "In contrast, S2 cells with Sec71 F713Y responded to a low concentration of BFA (5 μM)"

Please, answer here if the F713Y BFA-bodies have the same or similar structure compared to BFA-bodies characterized previously in WT cells. Were the size and density and even the marker distribution of these BFA bodies similar? This careful examination would be particularly important because there seems to be a difference in marker distribution between BFA bodies pointed with arrows on figure 2 L (right panel) and 2 N (left panel). Since authors use this line to investigate the mechanism of BFA body formation later, it is particularly important to clarify the similarities/differences here.

Reviewer's point "there seems to be a difference in marker distribution between BFA bodies pointed with arrows on figure 2 L (right panel) and 2 N (left panel) in the original manuscript. " might be caused by the difference of the strengths of green/red fluorescence.
In the original manuscript, the size of cells, F713Y treated with 5uM BFA (figure 2L, right panel, arrows) and WT treated with 50uM BFA (figure 2J, left panel, arrows) are too small to compare markers distribution in BFA body. Thus, we show new data in Figure 3 of revise manuscript. We compared the distribution of Golgi/RE markers (GM130, Golgin245, Rab11, GalT::EGFP, tdTomato::Rab6, tdTomato:: Rab11, ST::EGFP,) and Sec71 in F713Y BFA-bodies to WT BFA-bodies in new Figure 3 (and Figure 1C, D). These protein localizations are the same patterns in F713Y BFA-bodies and WT BFA-bodies. There is no obvious difference in the size of BFA-bodies in the wild type and Sec71^{F713Y} cells
.
Remark 5 - Page 8: "These results indicate that the impairment of Sec71 is necessary and sufficient for BFA-body formation. Thus, Sec71 is the only BFA-target responsible for BFA-body formation."

This concluding sentence is correct here and this is the place of that statement you made earlier (see Remark 3).

I agreed your indication and I keep the concluding sentence.

Remark 6 - Page 8: “In S2 cells with Sec71 M717L incubated with 25 μM BFA, Sec71 was concentrated at the center of BFA-bodies, accompanied by Rab11, however, Golgin245 was mostly found between GM130 and Sec71 (Fig. 3A, B lower panel and plots).”

First of all, there is and inconsistency between the labeling of the Figure 3, the figure legends and this sentence in results section. On Figure 3 itself there is no indication that we are looking at M717L mutant cells. According to the figure legends, cells on Fig3A are M717L mutant and cells on Fig3B are wild type S2. This sentence in the Results section says that both are M717L mutants.

It is not understandable why the authors used the M717L variant to demonstrate the localization of Sec71. They described in the preceding paragraph that M717L variant is resistant for BFA body formation (only 1% of cells show BFA-bodies after 50uM BFA treatment), but inconsistently with this, without any explanation, they used the very same resistant variant to show the localization of Sec71 (in this case Sec71M717L mutant) in BFA-bodies after treating the cells with 25uM (!) BFA. There are no statistics presented here. What was the percentage of M717L cells showing BFA-bodies with 25 uM BFA? If the formation of these structures is dependent on the dose of BFA, one can expect even less than 1% BFA-body formation in this case. Do the authors think or have evidence that the small number of BFA-bodies formed in M717L BFA resistant cells are accurately representing the structure of BFA-bodies in wild type cells and the accurate localization of Sec71?

We are very sorry that the previous manuscript had some serious errors. We actually used Sec71 F713Y S2 cells but not Sec71 M717L S2 cells in the experiments in Figure 3. We now showed the correct genotypes of cells in Figure 3. We also added some new results, which indicated the localization of Golgi markers and Sec71 in the wild type S2 cells, as we described in the answer for Remark 4.

Since they used lower BFA concentration here, this raises the question of whether the penetrance BFA-body formation is dose-dependent or not. Do the authors have data or are there data available in the literature that lower BFA concentration results is fewer BFA-bodies. It is not clear if 50 uM BFA is a plateau concentration above which you cannot see more BFA-body formation or it is a sort of balanced concentration where you can observe BFA-body formation but is not very toxic for the cells. Please, explain these points.

We investigated the dose-dependencies of WT, F713Y, M717L for BFA, and Figure 2L-N were replaced by new data sets, as we described in the answer for Remark 2.

Remark 7 – Page 8: “As the population of the wild type S2 cells responding to high concentration of BFA was limited, we used BFA-hypersensitive Sec71 F713Y S2 cells...”

More or less the same issue as Remark 6. Because of the lack of quantification, we don't know the frequency of BFA-body formation at this condition. We do know that F713Y responds to 5uM BFA to an extent as WT cells respond to 50uM BFA (i.e. roughly 20% of the cells show BFA body formation). How is the F713Y react to 25uM BFA? Please indicate if this increase in BFA concentration increases the frequency of BFA body formation (compared to 5 uM BFA used before). Is the structure of BFA bodies different by structure upon higher BFA concentration (i.e. BFA body size or density or even marker distribution change ?). I think it is crucial to clarify these details to justify the use of F713Y with 25uM BFA instead of WT with 50uM BFA.

The answer is also the same as Remark6. Biggest problem is in our mislabeling. Again, we are very sorry for the mistake in the previous manuscript. We used not Sec71 M717L S2 cells but used Sec71 F713Y S2 cells in the experiments for Figure 3.
In addition, as we already mentioned in the answer for Remark2, we now investigated the dose-dependencies of WT, F713Y, M717L for BFA and Figure 2L-N were replaced by new data sets.

Remark 8 – Page 8: “The merger and separation of TGNs was constantly repeated in BFA-untreated cells”
In order to more convincingly demonstrate the merging events of Golgi stacks, it would be great if authors could shoe a side view of Z-stacks of some images where the merging happens thus demonstrating that real merging/fusion event happened and not just superposition of two stacks in different depth which from a top view looks like merging.

Thank you so much for the important criticism. We included the new time lapse movies, Movie S2 and S3, which show both X-Y and Y-Z projections for BFA-untreated and BFA-treated cells. These movies clearly demonstrate the mergers and separations constantly repeated at TGNs.

Minor remark:

Discussion, page13, “...aggregated TGN/RE is central in BFA-bodies surrounded by Golgi stacks on the trans-side”

here I would suggest using the word “periphery” instead of “trans-side” to improve the clarity since “trans-side” in this context can have several different meanings.

We changed the sentence to “... aggregated TGN/RE is central and Golgi stacks locate at the periphery of BFA-bodies”.

Discussion, page 13, “...at low BFA concentration in high-penetration (Fig. 2J-M).”
Maybe an typo here. To be precise, you should refer to Fig. 2J-O.

Thank you very much. It is actually Fig. 2L-O. We fixed it.

Reviewer 2 Advance Summary and Potential Significance to Field:
Brefeldin A (BFA) has been used for decades as one of the most specific small molecule blockers of the secretory pathway. It inhibits the GEFs that activate the Arf GTPases on the Golgi. However, there are two main classes of Arf GEF and although BFA acts on both in many mammalian cells, in other species it acts on only one or the other. As a result, the structure of the Golgi following BFA treatment varies depending on the species. This paper reports a careful study of the effect of BFA in Drosophila cells. It had previously been shown that BFA blocks secretion, but the mechanism was unclear. This paper reports a series of well-designed and carefully quantified experiments that show clearly that in Drosophila S2 cultured cells, BFA acts solely on the Sec71 GEF of the trans Golgi network (TGN) and on no other cellular target. The authors go on to show that when S2 cells are treated with BFA then in a subset of cells the Golgi stacks coalesce to form a “BFA body” in which a central conglomeration of TGN and recycling endosome markers is surrounded by discrete elements containing early Golgi markers. The authors argue that this means that Arf activation is required for the TGN to separate from the rest of the stack. They also express a dominant negative version of Sec71 in photoreceptor cells and find that structures like BFA bodies appear with a concomitant block in delivery of proteins to the cell surface.

Overall this is a very high quality study which clearly demonstrates how BFA acts in Drosophila, whilst also providing new insight into the role of Arf in vivo. As such it is potentially suitable for publication, but there are a few minor technical issues that I feel need to be addressed, and also the authors’ interpretation of the mechanism behind Golgi body formation needs to be more clearly explained and justified. These points are outlined in the suggestions to authors.

Reviewer 2 Comments for the Author:
a) The authors should explain why Golgi stacks aggregate in only 20% of the treated cells.

We think the question how many percentages of cells make BFA-bodies is totally up to the definition of BFA-bodies. In some cells, Golgi stacks are gathering together, but not formed one big BFA-body. We included new Movie S3, which show 3D time-lapse observation of Golgi/TGNs in three dependent cells after BFA-addition. Two cells formed one big BFA-body in 60min but in the third cell, Golgi/TGNs gathered together, but only formed a couple of separated aggregates. In the statistics, such aggregates are not counted as BFA-bodies. We clarified what aggregates are counted for BFA-bodies in the Figure legend of the revised manuscript.
Another reason of low percentage of BFA-body formation is that we counted BFA-bodies in the single optical sections. As we compared the numbers of BFA-bodies in many conditions, taking Z-series of pictures for all conditions and counting BFA-bodies from individual Z-series movies are too much works and almost impossible. We used automated annotation to define cells and BFA-bodies to avoid biases in the statistics, which is too complicated to be done in 3D data. The z-projections made from Z-series stacks also have difficulty to distinguish BFA-bodies from the apparent aggregation of Golgi stacks locating on the different Z-sections.
Thus, in our statistics, we defined, the cells with focused Rab6 staining surrounded by GM130 staining are considered as with BFA-bodies. That would be the main reason of low rate of BFA-body formation.

b) The authors localise Rab11 using a fusion to Tq2. Have they tried using an antibody to look at the endogenous Rab11?

Antibody-staining of Rab11 is difficult because somehow it often gives high-background, but now we succeeded to show anti-Rab11 staining for both BFA-treated and untreated, wild type and Sec71^{F713Y} cells in new Figure 3C and 3D in revised manuscript.

c) Is it possible that the TGN and the Golgi-associated recycling endosome are the same thing? For instance Sec71-V5 looks very similar in distribution to Rab11. Also, do AP-1 and Rab11 co-localise?

We think the Golgi-associated recycling endosome (GA-RE) is very close to TGN, could be the same thing.,

However, because both RE and TGN have been defined in various ways, it is not easy to discuss the identity of TGN and GA-TGNs. We want to be cautious to make a conclusion about this issue, until we have information with much more markers and much higher resolution of RE and TGN.
The image we used in Fig2A of our first submission, it was true that the localization pattern of V5::Sec71 looks very similar to that of Rab11, however, we found it is not very typical when looked more samples. We concluded that V5::Sec71 colocalizes better with Rab6 than Rab11. Thus, we replaced the image of Fig 2A to the one better representing the colocalizations.
We have compared the localization of AP1 and Rab11 in our previous report (Fujii et al., 2020 jcs236935, Figure 2G): they colocalize well, but AP1 locates slightly more cis-side (closer to GalT).

d) The authors refer to the localisation of GalT::mTq2 on page 6, but do not mention which figure.

We indicated Figure number after the description of the localization of GalT::mTq2.

e) The authors state that BFA-bodies form in 22.6% of Sec71F713Y cells with 5 micromolar BFA. What is the frequency of BFA bodies in such cells treated with the 50 micromolar levels used elsewhere in the paper, or the 25 micromolar used with this particular mutant in Figure 4?

As we already motioned in the answer for the reviewer1 Remark 2 and Remark 7, we now investigated the dose-dependence of WT, F714Y, M717L for BFA and replaced the data in Figure 2L-N. This new experiment indicated that the proportion of Sec71^{F713Y} S2 cells with BFA-bodies does not dramatically increase by raising BFA dose from 5μM (27.2% (±1.7%)) to 30μM (28.0% (±3.5%)) or 50 μM (34.4% (±3.8%)) (Fig. 2L).

f) One key point is that I was confused by what the authors meant by "separation" when referring to Golgi stacks or TGNs. Do they mean the TGN separating away from the rest of the stack or do they mean the TGNs on two different stacks merging together to make one and then splitting apart to make two – ie scission? It is not clear to me as either seem possible (or even both at the same time). The authors also refer to "TGN/RE" separations which added to my confusion. It would help if the authors have a summary model figure in the Discussion to illustrate what they think is happening.

We are very sorry to confuse you by our unclear expression. We use the word 'separation" to mean the phenomenon that the merged TGNs of two different stacks are splitting apart to make two sets of Golgi stack/TGN". The phenomenon, TGN separating away from the rest of the Golgi stack is also occurred, but we did not count this type of phenomenon in Fig. 4C and D.
We also wonder to use the word 'scission', but the word 'scission' is used for the complete separation of the vesicle from the donner membrane after the budding process, and it is not clear that such vesicle scission occurs in the process of TGN separation.
We think the Golgi-associated recycling endosome (GA-RE) is very close to TGN, could be the same compartment. That is the reason we used TGN/RE in the discussion. (In the results, we used TGN separation, because we used a TGN marker, Rab6.)
In revised manuscript, we included the model figure as Fig. 8.

g) The movie showing BFA body formation suggests that the stacks move toward each other to form the structure. Does the BFA body form next to the centrosome? Is formation blocked by depolymerising microtubules?

There are no functional centrosomes at interphase in Drosophila cells, including S2 cells (Rusan and Rogers, 2009; Rogers et al., 2008), however, the S2 cells assemble functional centrosomes at mitosis. Thus, we compared BFA-body position with MT-asters formed from functional centrosomes at early prophase and found that BFA-body does not locate near MT-asters (Figure S2A upper panel). We also closely investigated BFA body positioning with microtubule-lattice at interphase (Figure S2A middle panel). We found that BFA-body itself is not tightly associated with MT, but some tdTomato::Rab6 positive tubules are extending along with MTs at the outside of BFA-body. We further examined the effect of MT-polymerization inhibitor, colchicine, on BFA-body formation, and found BFA-body could be formed without MT (Figure S2A lower panel). These results indicated MT is not necessary for the formation of BFA-bodies. We added these results on Figure S2A and also added a paragraph in the result section.

h) MPPE also seems to be present in the rhabdomeres as well as the Golgi (Figure 6). If this is correct the authors should note this and not simply state that MPPE is Golgi marker.

We added the following sentences in the legend of Figure 5B.
"Anti-MPPE antibody stains not only medial-Golgi but also the tip of the rhabdomeres. It is not known whether the latter staining shows the real MPPE localization or not."

i) There are also a few minor typos (eg marge instead of merge, and "action mechanism" instead of "mechanism of action"), and Drosophila is sometimes not in italics.

We fixed them.

Second decision letter

MS ID#: JOCES/2020/245571

MS TITLE: Sec71 separates Golgi stacks in Drosophila S2 cells

AUTHORS: Syara Fujii, Kazuo Kurokawa, Tatsuya Tago, Ryota Inaba, Arata Takiguchi, Akihiko Nakano, Takunori Satoh, and Akiko Kono Satoh
ARTICLE TYPE: Research Article

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

Reviewer 1

Comments for the author

A satisfactory revision was done. I suggest to accept for publication.

Reviewer 2

Comments for the author

The authors have done an excellent job of addressing my comments. They have added new data which makes their conclusions more robust, and they have added some useful extra discussion and a helpful summary figure. I enjoyed reading their response to my comments which was clearly written and very thorough. As such I am happy to recommend acceptance of this high quality study for publication in th Journal of Cell Science.