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Syntaxin 17, an ancient SNARE paralog, plays different and conserved roles in different organisms

Shun Kato, Kohei Arasaki, Natsuki Tokutomi, Yuzuru Imai, Tsuyoshi Inoshita, Nobutaka Hattori, Taeko Sasaki, Miyuki Sato, Yuichi Wakana, Hiroki Inoue and Mitsuo Tagaya
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MS TITLE: Syntaxin 17, an ancient SNARE paralog, plays different and conserved roles in different organisms

AUTHORS: Shun Kato, Kohei Arasaki, Natsuki Tokutomi, Yuzuru Imai, Tsuyoshi Inoshita, Nobutaka Hattori, Yuichi Wakana, Hiroki Inoue, and Mitsuo Tagaya
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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 which cover several of the conclusions of your manuscript. To address these concerns, I envisage that a substantial amount of work is needed. In this light, I fully understand if you would instead opt to submit your current results for publication elsewhere. Please let me know you decision.

Howevever, if you are willing to go down this route and 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 be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. 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 manuscript, the authors investigate the localizations and functions of the syntaxin-17 (Stx17) SNARE proteins from different organisms (human, fly and nematode). The approach they take is to express Drosophila melanogaster and Caenorhabditis elegans Stx17 proteins (dStx17 and cStx17, respectively) in human cells, and compare their localizations with that of human hStx17. The authors show that there are different localizations, and by making hybrid proteins, show that the C-terminal sequences of the 3 different Stx17 proteins are involved in localization. The majority of SNARE proteins have a C-terminal transmembrane (TM) domain that anchors them to membranes, through a post-translational insertion mechanism. It has been shown previously that Stx17 has a unique C-terminal membrane-binding region, consisting of two tandem transmembrane helices that interact with each other so as to remain in close proximity (using a “glycine zipper” mechanism). Most other SNAREs contain single C-terminal TM domains, and a dedicated machinery mediates post-translational insertion into membranes. The mechanism by which Stx17 proteins are inserted into membranes is not known, but perhaps uses the same machinery as for other tail-anchored proteins. The authors find that dStx17 is primarily cytosolic in mammalian cells, which may indicate that the membrane insertion mechanism is not functional on this heterologous dStx17. The C-terminal region of Stx17 is also important for mitochondrial localization, and the authors show that dStx17 fails to localize to mitochondria when expressed in mammalian cells.

The authors have previously shown that Stx17 depletion in mammalian cells causes mitochondrial elongation. Here they show that cStx17 but not dStx17 can complement this defect. The authors use a proximity ligation assay, and show that cStx17 gives a positive signal for interaction with Drp1, as does hStx17, but dStx17 does not. The authors also examine an autophagosomal phenotype of Stx17 depletion. They show that knockdown of Stx17 in mammalian cells results in a decrease in the number of LC-3 puncta in cells, and that expression of dStx17 but not cStx17 complements this defect. Stx17 depletion leads to a decrease in the number of lipid droplets in cells, and the authors show that expression of both dStx17 and cStx17 partially rescues this phenotype. dStx17 and cStx17 expression leads to an increase in lipid droplet number compared to Stx17 knockdown cells, but not to as high a level as hStx17 expression.

Comments for the author

Given the differences in localization between dStx17, cStx17 and hStx17 in mammalian cells, the mitochondrial, autophagosome and lipid droplet phenotypes observed could simply be a consequence of their different localizations. The authors provide some proximity ligation assay results to start examining the mechanisms involved, but in my opinion these results are not sufficient to support the conclusions drawn. In addition to proximity ligation assays, the authors should also carry out co-immunoprecipitation or other biochemical methods to assay protein-protein interactions.

However, the major criticism that I have of this study is the approach of expressing heterologous proteins in human cells, and the conclusions that the authors draw from these experiments. Notably, to conclude “that the localization of Stx17 is species-specific”, the authors need to determine the localization of each Stx17 in its own organism (dStx17 in Drosophila and cStx17 in C. elegans). What they have shown is the (not surprising) result that a protein adapted for function in flies or worms doesn’t localize or function in the same manner as its human homologue.
Detailed comments

1. Page 6. The authors state “starvation caused translocation of dStx17 from the cytosol to the microsome”. This is an incorrect statement, as no assay of translocation was performed. The authors should state that in their fractionation experiment, dStx17 was found in the microsome fraction in starved cells, whereas it was mostly cytososlic in cells grown under normal conditions.

2. Page 7 and Figure 2B. The fractionation results shown in Figure 2B for dStx17-deltaCtail are of very poor quality (anti-FLAG Western blot). A more convincing result should be shown.

3. Page 7. The authors should not refer to the “detachment of hStx17d from membranes and the association of hStx17c with membranes”. They are not assaying the dynamics of membrane association, but rather the steady-state localizations of these proteins. They should report their results, referring to cytosolic or membrane-bound localizations in cells, confirmed by fractionation experiments showing enrichment in cytosolic or membrane fractions.

4. Figure 2C. Why does the localization of LC-3 differ upon expression of the different constructs? In particular, the localization of LC-3 is very different in the cells shown in Figure 2B expressing either hStx17-deltaCtail or dStx17-deltaCtail. This difference should be explained, and its impact on colocalization determined. The authors here, and in other immunofluorescence experiments, should quantify their results in order to draw conclusions.

5. Figure 3C,D, Figure 4C,D and Figure 5C,D. Controls for the PLA experiments are missing. The authors should test cells depleted for the endogenous partner, as well as cells not expressing a FLAG-tagged protein.

Reviewer 2

Advance summary and potential significance to field

In this manuscript, Kato and his colleagues compared the localization and function of fly stx17 and nematode stx17 expressed in HeLa cells with human stx17. They found that these functional differences of stx17 may be caused by the different C-terminal structures of stx17. Their findings provide some new information for the evolution of stx17 in metazoa. The experiments are well done and the overall results are convincing.

Comments for the author

1. They only studied the effects of stx17 in flies and nematodes on human HeLa cells. In order to exclude potential artifact, they must provide evidence that these stx17 have the same function in flies and nematodes, respectively.

2. The authors claim that stx17 is not related to autophagy. However, it is not enough to detect the colocalization of stx17 and LC3, they should provide more evidence to verify the role of stx17 in autophagy. In addition, They should also study whether stx17 is really independent of autophagy in the nematode system.

3. In figures 1E, 2C and 2E, LC3 does not form dots in some images. It is unreasonable to compare the colocalization of stx17 and LC3 in these cells.

4. All siRNA knockout experiments should show knockout efficiency.

First revision

Author response to reviewers' comments

Point-by-point responses

Reviewer 1:

Comment 1 ---- Given the differences in localization between dStx17, cStx17 and hStx17 in mammalian cells, the mitochondrial, autophagosome and lipid droplet phenotypes observed could simply be a consequence of their different localizations. The authors provide some proximity ligation assay results to start examining the mechanisms involved, but in my opinion these results are not sufficient to support the conclusions drawn. In addition to proximity ligation assays, the authors should also carry out co- immunoprecipitation or other biochemical methods to assay protein-protein interactions.

Reply ---- Following this suggestion, we have performed immunoprecipitation experiments. The results are consistent with those of proximity ligation assay. GFP-Drp1 (K38A), which is defective in hydrolysis but retains some GTP-binding ability, co-precipitated with hStx17 (human Stx17) and cStx17 (C. elegans Stx17), but much less with dStx17 (D. melanogaster Stx17) (Fig. 3E). Endogenous Atg14L co-precipitated with hStx17 and dStx17, but much less with cStx17 (Fig. 4E). GFP-ACSL3, but not GFP-ACSL3 lacking the GATE domain, which is required for binding to hStx17 (Kimura et al., 2018, J. Lipid Res.), co-precipitated with all the three species (Fig. 5E).

Comment 2 ---- However, the major criticism that I have of this study is the approach of expressing heterologous proteins in human cells, and the conclusions that the authors draw from these experiments. Notably, to conclude "that the localization of Stx17 is species-specific", the authors need to determine the localization of each Stx17 in its own organism (dStx17 in Drosophila and cStx17 in C. elegans). What they have shown is the (not surprising) result that a protein adapted for function in flies or worms doesn't localize or function in the same manner as its human homologue.

Reply ---- We have expressed hStx17, dStx17 and cStx17 in *Drosophila* S2 cells and examined their localization without or with starvation treatment. The results were essentially the same as those observed in HeLa cells: In fed cells, hStx17 and cStx17 were found almost exclusively in the membrane fraction, whereas dStx17 was largely cytosolic (Fig. 6B, top). Upon starvation, much more dStx17 was found in the membrane fraction (Fig. 6B, bottom). Endogenous dStx17 was also observed mostly in the cytosolic fraction in fed cells, whereas it was found in the membrane fraction in starved cells (Fig. 6A).

We tried to express GFP-cStx17 in *C. elegans*, but unfortunately, no expression was observed. This may imply that overexpressed GFP-cStx17 may be toxic for individuals.

Detailed comment 1 (Page 6) ---- The authors state "starvation caused translocation of dStx17 from the cytosol to the microsome". This is an incorrect statement, as no assay of translocation was performed. The authors should state that in their fractionation experiment, dStx17 was found in the microsome fraction in starved cells, whereas it was mostly cytosolic in cells grown under normal conditions.

Reply ---- We have modified the statement as suggested (page 6, second paragraph).

Detailed comment 2 (Page 7 and Figure 2B) ---- The fractionation results shown in Figure 2B for dStx17-deltaCtail are of very poor quality (anti-FLAG Western blot). A more convincing result should be shown.

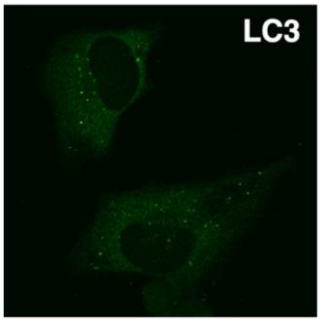
Reply ---- We repeated the experiment, and the result of which is shown in Fig. 2B. Clearly, dStx17ΔC-tail was found almost exclusively in the membrane fraction.

Detailed comment 3 (Page 7) ---- The authors should not refer to the "detachment of hStx17d from membranes and the association of hStx17c with membranes". They are not assaying the dynamics of membrane association, but rather the steady-state localizations of these proteins. They should report their results, referring to cytosolic or membrane- bound localizations in cells, confirmed by fractionation experiments showing enrichment in cytosolic or membrane fractions.

Reply ---- We have modified the statement as suggested (page 7, second paragraph).

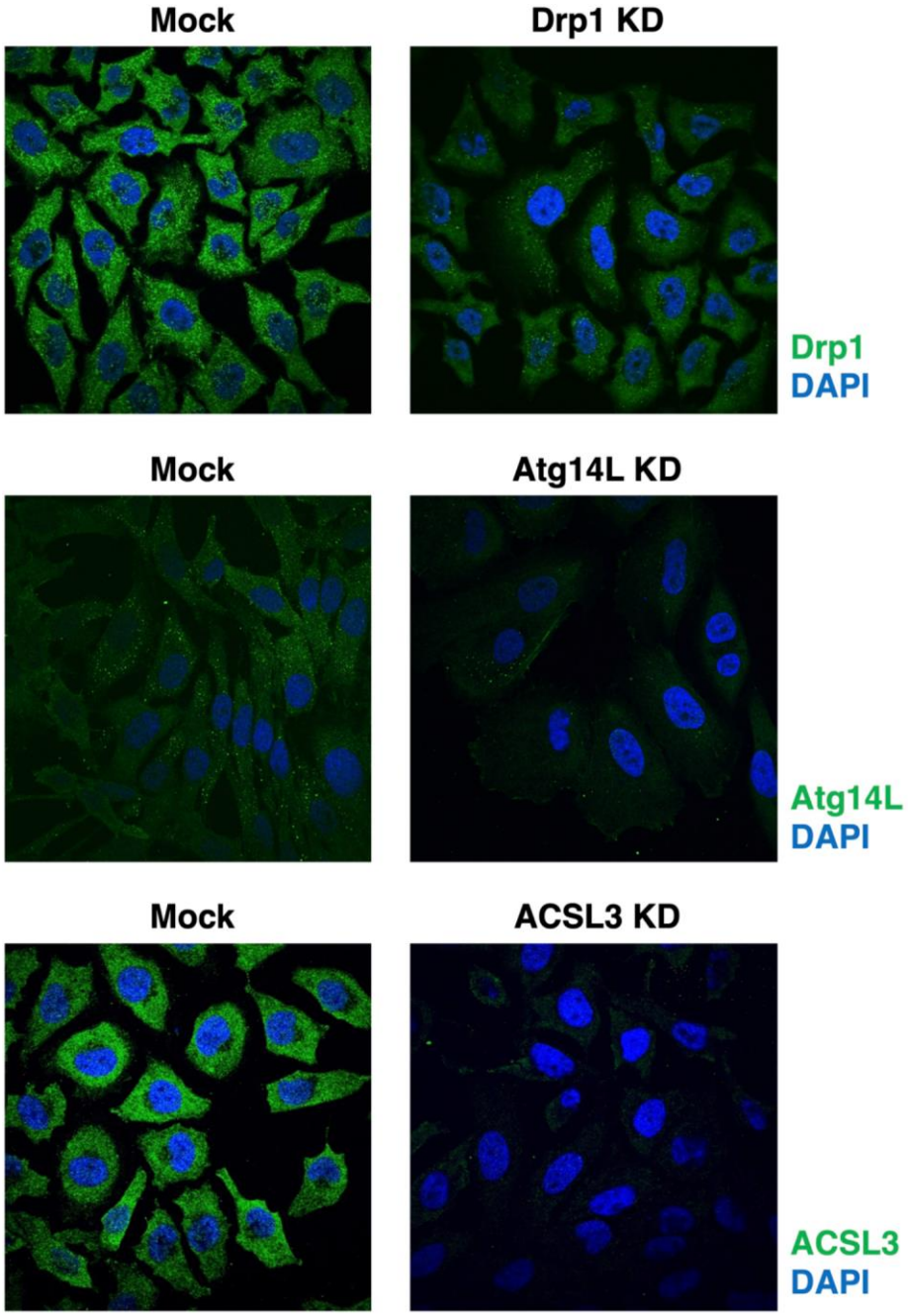
Detailed comment 4 (Figure 2C) ----Why does the localization of LC-3 differ upon expression of the different constructs? In particular, the localization of LC-3 dots is very different in the cells shown in Figure 2B expressing either hStx17-deltaCtail or dStx17- deltaCtail. This difference should be explained, and its impact on colocalization determined. The authors here, and in other immunofluorescence experiments, should quantify their results in order to draw conclusions.

Reply ---- Overexpression of Stx17 involved in autophagosome formation increased the size of LC3 and affected LC3 localization. The right figure shows a typical immunofluorescence image of cells not expressing Stx17. As suggested, we have quantified the data and shown the result in Fig. 2C, bottom, and replaced fluorescence images with better definition. The quantification data have been also added in Fig. 1E, bottom right.

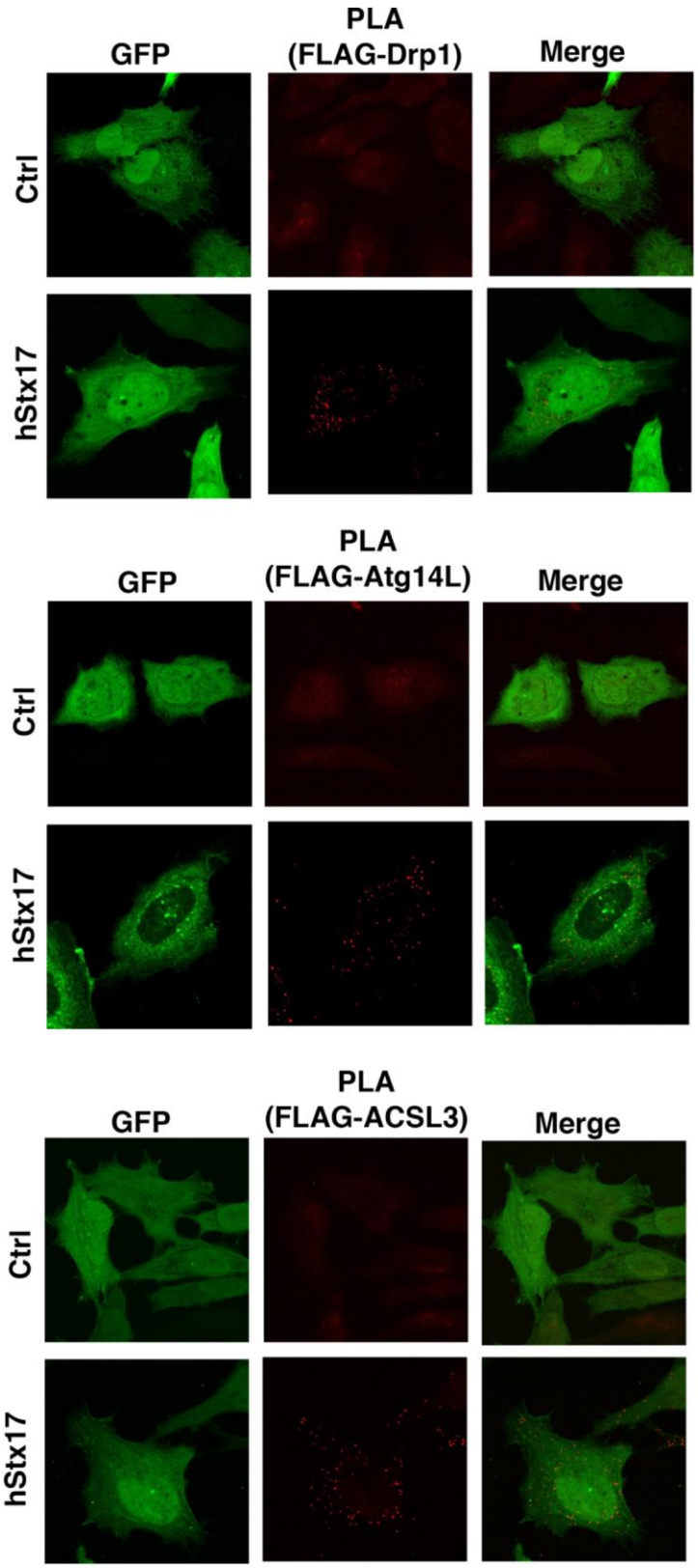


Detailed comment 5 (Figure 3C,D, Figure 4C,D and Figure 5C,D.) ---- Controls for the PLA experiments are missing. The authors should test cells depleted for the endogenous partner, as well as cells not expressing a FLAG-tagged protein.

Reply ---- The specificity of the antibodies used for PLA is shown in the figure below. Immunoreactivity almost completely disappeared upon knockdown of each protein.



Moreover, no PLA signals were observed when FLAG-Stx17 was not transfected, as shown in the figure below (Ctrl).



We have stated the above results in page 20, first paragraph, as follows.
“The specificity of antibodies used for PLA was confirmed by reduction in immunofluorescence signal in cells depleted of antigens and in PLA signal in cells without expression of FLAG-tagged

Stx17 (data not shown). ”

Reviewer 2:
Comment 1 ----They only studied the effects of stx17 in flies and nematodes on human HeLa cells. In order to exclude potential artifact, they must provide evidence that these stx17 have the same function in flies and nematodes, respectively.
Comment 2 ----The authors claim that stx17 is not related to autophagy. However, it is not enough to detect the colocalization of stx17 and LC3, they should provide more evidence to verify the role of stx17 in autophagy. In addition, they should also study whether stx17 is really independent of autophagy in the nematode system.

Reply ---- As Stx17 was reported to participate in autophagy (Takats et al., 2013, J. Cell Biol.), we have examined whether Stx17 does not regulate mitochondrial division. The results have demonstrated that Stx17 knockdown in S2 cells (Fig. 6D) or Stx17 ablation in *Drosophila* (Fig. 6E) does not affect mitochondrial length, although mitochondrial cristae morphology is disrupted upon Stx17 ablation (Fig. 6E and Sugo et al., 2018, EMBO J.).

Regarding *C. elegans*, we have shown that loss of Stx17 does not disrupt allophagy (Fig. 7), a type of autophagy triggered by fertilization to remove sperm mitochondria (Sato and Sato, 2011, Science). Unfortunately, during the time frame of this revision, we could not obtain evidence for the involvement of Stx17 in mitochondrial division in nematode. Further study is necessary to address this point. We state this at the end of the discussion section.

Overall, the non-participation of fly Stx17 and nematode Stx17 in mitochondrial division and autophagy, respectively, which was predicted from the data obtained using heterologous expression of Stx17 species in HeLa cells, has been demonstrated in individual organisms. Of note, in HeLa cells nematode Stx17 neither colocalizes with LC3, nor interact with Atg14L (Fig. 4C-E).

Comment 3 ---- In figures 1E, 2C and 2E, LC3 does not form dots in some images. It is unreasonable to compare the colocalization of stx17 and LC3 in these cells.

Reply ---- We have replaced the images to ones with better definition and added the quantification data.

Comment 4 ---- All siRNA knockout experiments should show knockout efficiency.

Reply ---- The knockdown efficiency data are shown in Fig. 3A and Fig. 6C.

Second decision letter

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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

Advance summary and potential significance to field

In this manuscript, the authors investigate the localizations and functions of the syntaxin-17 (Stx17) SNARE proteins from different organisms (human, fly and nematode). The approach they take is to express human, fly and nematode Stx17 proteins in human cells and in flies, and compare their localizations. The authors show that there are different localizations, and by making hybrid proteins, show that the C-terminal sequences of the 3 different Stx17 proteins are responsible for these differences. The majority of SNARE proteins have a C-terminal transmembrane (TM) domain that anchors them to membranes, through a post-translational insertion mechanism. It has been shown previously that Stx17 has a unique C-terminal membrane-binding region, consisting of two tandem transmembrane helices that interact with each other so as to remain in close proximity (using a “glycine zipper” mechanism). Most other SNAREs contain single C-terminal TM domains, and a dedicated machinery mediates post-translational insertion into membranes. The mechanism by which Stx17 proteins are inserted into membranes is not known, but perhaps uses the same machinery as for other tail-anchored proteins. The authors find that fly Stx17 is primarily cytosolic in mammalian cells, which may indicate that the membrane insertion mechanism is not functional on this heterologous fly Stx17. The C-terminal region of Stx17 is also important for mitochondrial localization, and the authors show that fly Stx17 fails to localize to mitochondria when expressed in mammalian cells. In contrast, nematode Stx17 localizes to mitochondria and facilitates mitochondrial division, but does not affect autophagy. In summary, the authors show that fly Stx17 is not involved in mitochondrial division and that nematode Stx17 does not function in autophagy, unlike human Stx17, providing insight into the evolution of this important SNARE protein in metazoans.

Comments for the author

The authors have fully addressed my comments, and I am satisfied that this revised version of the manuscript is suitable for publication.

Reviewer 2

Advance summary and potential significance to field

In this revised manuscript, the authors have conducted additional experiments using S2 cells and C.elegans.The results obtained from these experiments confirm their conclusions in Hela cells. In general, the authors have solved my main concern about this story, and the manuscript can be considered for publication in JCS.

Comments for the author

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