EMBO reports

# Extended synaptotagmin regulates contact site structure and lipid transfer function in vivo

Vaisaly Nath, Shirish Mishra, Bishal Basak, Deepti Trivedi, and Raghu Padinjat
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## Review Timeline:

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| Submission Date: | 20th Feb 20 |
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## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

## 1st Editorial Decision 6th Mar 2020

Dear Prof. Padinjat,

Thank you for the submission of your research manuscript to our journal, which was now seen by two referees, whose reports are copied below.

While referee #1 appreciates the analysis, he/she also finds that demonstration of a role of dEsyt in maintenance and function of Drosophila photoreceptors does not constitute a sufficient conceptual advance for publication here, because the mechanism by which dEsyt regulates MCS was previously demonstrated by multiple studies. However, referee #2 finds that demonstration of the functional relevance is of sufficiently broad interest and supports that a revised version should be considered. Given the discrepancy in the referee views, I involved a third expert. The expert notes that 'I think it has impact to show that in a real cell responding to normal physiological stimuli, the loss of the Esyt really does matter both to the survival of the cell and the structure of the ER PM contacts.' He/she further notes 'I have more of sense that the Esyt actually matters after reading Nath et al. Many people will already have assumed its importance from the earlier literature, but having a clear idea of just how much it matters (and how little) in a cell more normal than a HeLa cells is going to be of interest to the lipid transfer, membrane contacts worlds.' The advisor also raised some concerns regarding statistical analyses, which you can find below and need to be addressed along with the referee concerns.

Given the positive recommendations of referee #2 and our expert advisor, we have decided to invite you to revise your manuscript with the understanding that the referee and the 3rd expert's concerns (as in their reports below) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1. A data availability section providing access to data deposited in public databases is missing (where applicable).
2. Your manuscript contains statistics and error bars based on n=2 or on technical replicates. Please use scatter plots in these cases.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes

a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14693178/authorguide#transparentprocess
You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines ().

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) We would also encourage you to include the source data for figure panels that show essential data.

Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple

images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

8) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

9) Please make sure to include a Data Availability Section before submitting your revision - if it is not applicable, make a statement that no data were deposited in a public database. Primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see ).

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

# Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

10) Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates.
Please also include scale bars in all microscopy images.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if

you have questions or comments regarding the revision.

Yours sincerely,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

The 3rd expert:

One additional issue, however leapt out at me in Raghu's manuscript and that is the incorrect use of the phrase "Not Significant" or "no difference" and absence of actual p values for experiments marked as "ns". In several of these (for example Figures 2B and C, 3F, 5B, S5A) the average or mean values of control and mutant are certainly different, but perhaps they are p =0.1 rather than 0.05. But of course even a p=0.1 is 10 times more likely to be a real difference than a chance event. To say "no difference" is factually wrong and such differences should not be ignored just because there is a lot of scatter in the data. If the reader is given the actual p value (and not just ns vs. *) they can decide for themselves how much or how little confidence to place in the data and the consequent interpretations. The difference between ns and * can be as paltry as 0.051 and 0.049 which is why we need the real p values.

Referee #1:

This paper on the sole tricalbin/Esyt in drosophila photoreceptors shows that the deleted animals have a retinal degeneration phenotype on exposure to light over many days, similar to that in other lipid handling proteins - DG kinase RdgA and the lipid transfer protein RdgB. It shows a functional interaction between Esyt and RdgB. An ultrastructural correlate is nicely described: tethering of sub-rhabdomeric ER cisternae with rhabdomeric PM is lost during ~14 days of light exposure if Esyt is missing. This is (like rdgA and rdgB mutants) epistatic to norpA mutation, as PLC is required to activate TRP in response to light. This is also similar to (less extreme than) RdgB mutations.

The link between RdgB and Esyt is clear, but it has been made before. Here, Esyt is also implicated in ER-PM tethering. While tethering is an obvious concept that seems to have face validity, it is only one means by which Esyt might act to support the function of RdgB.

To compare the paper against the criteria for acceptance in EMBO Reports, I will start with physiological relevance. The functional insight parallels the paper by Chang et al (2013) that the authors reference. Making the Esyt-RdgB link in a living animal does have more physiological relevance compared to tissue culture cells, however I would say that this is very much to be expected from the first paper, and that the criterion is not met purely by repeating the experiment in a cell type that uses pathways that were previously tested.

This leaves the question of whether the work produces a notable conceptual advance. To do this, there needs to be more understanding of how Esyt interacts with RdgB. A comprehensive genetic analysis of Esyts was published in 2019 by another group on Esyts in a different model system: yeast (Hoffman et al., Dev Cell; PMID 31743663), which admittedly has no RdgB-like LTP. This paper

clearly demonstrated that Esyts are required for a wide number of pathways. Many of these were unexpected. A key finding was the lack of involvement of the SMP domain for most functions, so maybe the role of this lipid transfer protein element is not as has been assumed.

This paper should analyze Esyt at least to that level. Depending on those results, it might still not make much of a mechanistic advance, and could be best suited for a specialist membrane cell biology journal. To be acceptable in EMBO Reports, the issue of how Esyt affects ER-PM MCS size over time (Fig 5/6 - see below) should be examined and understood better. Without that, the MS is not a strong candidate for a journal with a wide readership.

Major results:
Fig 2H: the authors produce a good platform to reconstitute Esyt with mutants, for example in the SMP or C2 domains. Ideally these will be point mutants: SMP can be mutated to "fill in" the lipid binding site (see PMID 28011845), C2 can lose its Ca2+ chelating residues. Domain deletions were good enough for Hoffman, but that is more risky approach.

Fig 3: partly confirms finding of Kim et al 2013 that Esyt is required for RDGB function

Fig 5/6: ER-rhabdomere contact lost over 14d light exposure. This parallels the phenotype of lack of full RDGB function. It is altered by inhibiting PLCb, without which a moderate amount of contact is preserved independent of Esyt, indicating that the Esyt/RDGB axis is needed to maintain the cells after signaling through PLC/Ca2+, which is not surprising.
For these data sets, show how loss of Esyt affects other ER-PM contact zones: in the photoreceptors but not sub-rhabdomeric, in adjacent cells, in other cell types that might be expected to have similar DAG production - including the neurons looked at by Kikuma et al. (2017). The (?gradual - should be tested) reduction ER-PM MCS is potentially a strong finding, but as things stand it might have multiple explanations. Re-expressing point mutants of dEsyt is one way to get at the answer. It would also be good include a second level of experimentation to confirm understanding. For example, if an SMP mutant does rescue, implying tethering by dEsyt, then a second level of experimental evidence would be to attempt rescue by a heterologous tether (as other groups are trying to do: see www.biorxiv.org/content/10.1101/657999v2)

Fig S6: this is convincing EM evidence that Esyt can tether rhabdomeric PM to ER. Could be in the main MS.
As with deletion EM experiments, show effects throughout the animal.

Minor points:
Family tree (Fig 1A): this makes a bold claim that the Drosophila protein is more similar to its yeast homologs than the human ones. Bold claims need bold supporting evidence. I find this whole tree dubious, from the position of dEsyt to the colours and the scale. To get accurate rooting as many related proteins have to be included as possible. Just starting with seven will not get the right result. A BLAST search I carried out on the web produces vertebrate hits with e-value 10^-103; while the top fungal hit had e-value 10^-23.

Separation of R7 data from R1-6 (Figs 5 & 6) needs to be fully explained in the text. As it is the differences are briefly described without any comment.

Referee #2:

In this study, Nath et al. explore the physiological role of extended synaptotagmin (Esyt) in Drosophila. ESyts represent a family of proteins conserved form yeast to man that function as lipid transport proteins at ER-plasma membrane (PM) contact sites. While phenotypes associated with Esyt knockouts in both yeast and mammalian systems have proven elusive and/or minimal, Nath et al. demonstrate that knockout of the only drosophila dEsyt results in defects in retinal phototransduction, a process known to rely on ER-PM cross-talk. Using a combination of electroretinogram recording, fluorescence and electron microscopy they show that absence of dEsyts results in retinal degeneration. They also demonstrate that, prior to degeneration of photoreceptors, absence of dEsyt exacerbates phenotypes associated with defective function of the lipid transfer protein RDGB in phototransduction, and show that correct localization of RDGB at ER-PM contact sites depends on the presence of dEsyt. I consider this manuscript appropriate for publication in EMBO Report for two reasons. This study is the first to show convincing evidence for a role of an Esyt in a physiological process in an intact organism. Second, the functional connection of dEsyt to RDGB shown here strongly supports a role of dEsyt as a lipid transporter and provides new clues about its precise mechanism of action.

Specific comments:

The dEsytKO and the rdgB9 retinal degeneration studies were performed under constant bright light. RDGB mislocalization in dEsytKO flies was also observed under constant illumination. However, the TEM studies of MCS ultrastructure were performed on dark reared flies. What was the rationale behind this choice of experimental protocol?

Any explanation on why the RDGB staining is more punctate at day1 in dESytKO eyes (Fig. 4D)?

The EM images are difficult to interpret. Why does the ER appear black in EM images? And why in some images does it have a bead-like appearance. Changes to the text and to the figure legends to better describe the EM micrographs will be helpful.

Minor comments:

Further explanation of the soD mutation is warranted

Phalloidin staining of rhabdomeres in the Figure 4B WT condition appears diffuse. A better image would be desirable

In the text Figure 4B is discussed prior to 4A. Please re-order either the text or the figure sequence

## Response to reviewer comments: Nath et.al

We thank the reviewers for their comments and feedback. Responses to individual comments are presented below. Changes in the revised manuscript are highlighted in yellow.

### The 3rd expert:

**One additional issue, however leapt out at me in Raghu's manuscript and that is the incorrect use of the phrase "Not Significant" or "no difference" and absence of actual p values for experiments marked as "ns". In several of these (for example Figures 2B and C, 3F, 5B, S5A) the average or mean values of control and mutant are certainly different, but perhaps they are p =0.1 rather than 0.05. But of course even a p=0.1 is 10 times more likely to be a real difference than a chance event. To say "no difference" is factually wrong and such differences should not be ignored just because there is a lot of scatter in the data. If the reader is given the actual p value (and not just ns vs. *) they can decide for themselves how much or how little confidence to place in the data and the consequent interpretations. The difference between ns and * can be as paltry as 0.051 and 0.049 which is why we need the real p values.**

***Response:*** We thank the expert for bringing this point to our notice; in several of the graphs where the p-value is not significant, the average or mean values of control and mutant are different and the p-value ranges between 0.05 and 0.1. The required changes are made and the respective p-values are presented along with the significance statement in all graphs.

### Referee #1:

This paper on the sole tricalbin/Esyt in drosophila photoreceptors shows that the deleted animals have a retinal degeneration phenotype on exposure to light over many days, similar to that in other lipid handling proteins - DG kinase RdgA and the lipid transfer protein RdgB. It shows a functional interaction between Esyt and RdgB. An ultrastructural correlate is nicely described: tethering of sub-rhabdomeric ER cisternae with rhabdomeric PM is lost during ~14 days of light exposure if Esyt is missing. This is (like rdgA and rdgB mutants) epistatic to norpA mutation, as PLC is required to activate TRP in response to light. This is also similar to (less extreme than) RdgB mutations.

The link between RdgB and Esyt is clear, but it has been made before. Here, Esyt is also implicated in ER-PM tethering. While tethering is an obvious concept that seems to have face validity, it is only one means by which Esyt might act to support the function of RdgB.

***Response:*** We agree with the reviewer that Esyt could support the function of RDGB in many possible ways. We would like to reiterate that nowhere in our manuscript have we stated that Esyt functions as a tether in this model system. We have noted and placed on record the mis-localization of RDGB protein from ER-PM MCS and also the disruption of the MCS upon loss of dEsyt in these cells. There could be many mechanisms by which this phenotype arises, loss of tethering between the two membranes being one of them.

To compare the paper against the criteria for acceptance in EMBO Reports, I will start with physiological relevance. The functional insight parallels the paper by Chang et al (2013) that the authors reference. Making the Esyt-RdgB link in a living animal does have more physiological relevance compared to tissue culture cells, however I would say that this is very much to be expected from the first paper, and that the criterion is not met purely by repeating the experiment in a cell type that uses pathways that were previously tested.

***Response:*** The paper of Chang et.al *Cell Reports*, 2013 reports that when overexpressed in cultured mammalian cells Nir2 (the mammalian ortholog of RDGB) is recruited to ER-PM contact sites. It is also reported that siRNA depletion of ESyt1 reduces the accumulation of Nir2 at ER-PM contact sites in these cultured cells. However, we would like to highlight the following studies published independent of the paper of Chang et.al 2013:

(1) loss of Esyt function in cultured mammalian cells does not impact $PIP_2$ turnover during PLCβ signaling and that depletion of Esyt does not impact store-operated calcium influx, a key output of PLCβ activation (Giordano, et.al, *Cell*, 2013)
(2) It has been published by Saheki, et.al *Nat. Cell. Biol*, 2016 that in Esyt KO cells, there is no defect in the kinetics of $PIP_2$ turnover during PLC activation

Therefore, we are of the opinion that it may not be appropriate to state that *"I would say that this is very much to be expected from the first paper"*. The value of our study is that it is a demonstration of the importance of endogenous Esyt in supporting the lipid transfer function of RDGB *in vivo*.

This leaves the question of whether the work produces a notable conceptual advance. To do this, there needs to be more understanding of how Esyt interacts with RdgB. A comprehensive genetic analysis of Esyts was published in 2019 by another group on Esyts in a different model system: yeast (Hoffman et al., Dev Cell; PMID 31743663), which admittedly has no RdgB-like LTP. This paper clearly demonstrated that Esyts are required for a wide number of pathways. Many of these were unexpected. A key finding was the lack of involvement of the SMP domain for most functions, so maybe the role of this lipid transfer protein element is not as has been assumed.

This paper should analyse Esyt at least to that level. Depending on those results, it might still not make much of a mechanistic advance, and could be best suited for a specialist membrane cell biology journal. To be acceptable in EMBO Reports, the issue of how Esyt affects ER-PM MCS size over time (Fig 5/6 - see below) should be examined and understood better. Without that, the MS is not a strong candidate for a journal with a wide readership.

***Response:*** We are aware of the work of Hoffman et al., Dev Cell; PMID 31743663. On careful perusal of that manuscript, the following points emerge:

(i) Consistent with multiple previous studies, it has been explicitly stated by Hoffmann et.al (on p492 of their paper) that deletion of all three tricalbins in yeast does not have a measurable phenotype *in vivo*.

(ii) The analysis of Esyt/tricalbin domain requirement has been done in the context of synthetic lethality with mutants of other yeast genes in a range of pathways and processes; surprisingly none of them involve lipid transfer function. Remarkably, the insightful conclusion arrived from the analysis of Hoffman et.al is that none of the domains of tricalbins are required for any of these synthetic lethality interactions, i.e protein can do away with each of its domains. We feel this reflects the complex nature of their experimental design (which is explained on p494 of their paper) and will require careful thought before further insights are inferred or attempts to repeat this in other models are warranted.

(iii) The most important insights from the work of Hoffman and colleagues is that they provide a detailed ultrastructural analysis of contact sites in yeast using both EM and correlative microscopy.

By contrast, our study is a focused attempt to establish the importance of Esyt for maintaining ER-PM MCS and lipid transfer in an *in vivo* context using a model organism with a single Esyt gene. We describe a phenotype for the knockout of the single *Drosophila* Esyt gene, present evidence for its contribution to ER-PM MCS stability *in vivo* and its role in modulating the function of RDGB *in vivo*.

Therefore we feel that it is not appropriate to compare or benchmark our manuscript against the studies of Hoffmann et.al.

1. **Major results:**

**Fig 2H: the authors produce a good platform to reconstitute Esyt with mutants, for example in the SMP or C2 domains. Ideally these will be point mutants: SMP can be mutated to "fill in" the lipid binding site (see PMID 28011845), C2 can lose its Ca2+ chelating residues. Domain deletions were good enough for Hoffman, but that is more risky approach.**

***Response:*** We thank the reviewer for these suggestions and for appreciating that our model system, where loss of the single Esyt has clear phenotypes, offers a suitable platform for the functional analysis of the domains of dEsyt. However, we feel that these experiments, if they are to be done in a meaningful manner, would require a huge amount of work and substantial time to accomplish. The outcomes, namely the molecular mechanisms by which Esyt works are beyond the scope of the core message of this manuscript. The core message of this paper is that loss of Esyt has clear phenotypic consequences *in vivo* including on ER-PM MCS stability as well as the function of RDGB, a lipid transfer protein.

**Fig 3: partly confirms finding of Kim et al 2013 that Esyt is required for RDGB function**

***Response***: We could not locate a paper in Pubmed by the author Kim in 2013 that has analysed Esyt and RDGB function.

**Fig 5/6: ER-rhabdomere contact lost over 14d light exposure. This parallels the phenotype of lack of full RDGB function. It is altered by inhibiting PLCb, without which a moderate amount of contact is preserved independent of Esyt, indicating that the Esyt/RDGB axis is needed to maintain the cells after signaling through PLC/Ca2+, which is not surprising. For these data sets, show how loss of Esyt affects other ER-PM contact zones: in the photoreceptors but not sub-rhabdomeric, in adjacent cells, in other cell types that might be expected to have similar DAG production - including the neurons looked at by Kikuma et al. (2017).**

***Response:*** Our manuscript is a focused study attempting to establish the role of dEsyt in *Drosophila* photoreceptors, a cell type in which the function of RDGB, a lipid transfer protein at ER-PM MCS is well established through prior analysis. While it is undoubtedly interesting to study ER-PM MCS in other cell types in *Drosophila*, we feel this is beyond the scope of this manuscript.

**The (gradual - should be tested) reduction ER-PM MCS is potentially a strong finding, but as things stand it might have multiple explanations. Re-expressing point mutants of dEsyt is one way to get at the answer. It would also be good include a second level of experimentation to confirm understanding. For example, if an SMP mutant does rescue, implying tethering by dEsyt, then a second level of experimental evidence would be to attempt rescue by a heterologous tether (as other groups are trying to do: see www.biorxiv.org/content/10.1101/657999v2)**

***Response:*** It should be reiterated once again that our manuscript does not make a claim that dEsyt functions as a tether. We thank the reviewer for this interesting suggestion. This would be a valuable experiment for a manuscript that seeks to explain the molecular mechanism by which Esyt protein works at ER-PM contact sites.

**Fig S6: this is convincing EM evidence that Esyt can tether rhabdomeric PM to ER. Could be in the main MS.**

***Response:*** We thank the reviewer for their suggestion

**Minor points:**
**Family tree (Fig 1A): this makes a bold claim that the Drosophila protein is more similar to its yeast homologs than the human ones. Bold claims need bold supporting evidence. I find this whole tree dubious, from the position of dEsyt to the colours and the scale. To get accurate rooting as many related proteins have to be included as possible. Just starting with seven will not get the right result. A BLAST search I carried out on the web produces vertebrate hits with e-value 10^-103; while the top fungal hit had e-value 10^-23.**

***Response:*** This has been rectified. The phylogenetic tree has now been redone with 22 sequences including human, mouse, rat, *Xenopus*, *Drosophila*, worm and yeast.

**Separation of R7 data from R1-6 (Figs 5 & 6) needs to be fully explained in the text. As it is the differences are briefly described without any comment.**

***Response:*** Rectified. The explanation on why R7 data is plotted separate from R1-R6 is explained in the text.

## Referee #2:

In this study, Nath et al. explore the physiological role of extended synaptotagmin (Esyt) in Drosophila. ESyts represent a family of proteins conserved form yeast to man that function as lipid transport proteins at ER-plasma membrane (PM) contact sites. While phenotypes associated with Esyt knockouts in both yeast and mammalian systems have proven elusive and/or minimal, Nath et al. demonstrate that knockout of the only drosophila dEsyt results in defects in retinal phototransduction, a process known to rely on ER-PM cross-talk. Using a combination of electroretinogram recording, fluorescence and electron microscopy they show that absence of dEsyts results in retinal degeneration. They also demonstrate that, prior to degeneration of photoreceptors, absence of dEsyt exacerbates phenotypes associated with defective function of the lipid transfer protein RDGB in phototransduction, and show that correct localization of RDGB at ER-PM contact sites depends on the presence of dEsyt. I consider this manuscript appropriate for publication in EMBO Report for two reasons. This study is the first to show convincing evidence for a role of an Esyt in a physiological process in an intact organism. Second, the functional connection of dEsyt to RDGB shown here strongly supports a role of dEsyt as a lipid transporter and provides new clues about its precise mechanism of action.

**Specific comments:**

**The dEsytKO and the rdgB9 retinal degeneration studies were performed under constant bright light. RDGB mislocalization in dEsytKO flies was also observed under constant illumination. However, the TEM studies of MCS ultrastructure were performed on dark reared flies. What was the rationale behind this choice of experimental protocol?**

***Response:*** During initial studies with *dEsyt*$^{KO}$ we performed retinal degeneration analysis under two experimental conditions, namely constant dark and constant light. These experiments revealed that *dEsyt*$^{KO}$ showed no retinal degeneration upto 14 days in constant dark but does so in constant light. Hence subsequent work and the RDGB localization studies were performed in constant light when a clear phenotype was present. This is typical of some *Drosophila* retinal degeneration mutants where degeneration in the dark is slow and is accelerated by light or where the phenotype under consideration is light dependent. This literature is reviewed in

Raghu, P***,** Yadav, S and Mallampati, N. Lipid signalling in Drosophila photoreceptors. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids. Vesicular Transport. 2012;* 1821(8):1154-65.

In the case of *rdgB*$^{9}$;*dEsyt*$^{KO}$ double mutants as well, retinal degeneration studies were carried out under all three experimental conditions of constant dark, 12 hour light/dark cycle and constant light. Acceleration of retinal degeneration in *rdgB*$^{9}$ by dEsyt$^{KO}$ was seen in all three experimental conditions with constant light showing maximum enhancement. For the double mutants of *rdgB*$^{9}$;*dEsyt*$^{KO}$, retinal degeneration in constant dark is shown in Fig 3A.

It is important to appreciate that even though *rdgB*$^{9}$ is a light dependent degeneration, it too will degenerate in the dark and show phenotypes, albeit at a much slower rate. This is because, as previously demonstrated, even in the dark, *Drosophila* photoreceptors show high basal PLC activity that is further enhanced by illumination. This is described in:

Hardie, R.C, Gu, Y., Martin, M., Sweeney, S.T and Raghu, P. In Vivo light-induced and basal phospholipase C activity in Drosophila photoreceptors measured with genetically targeted phosphatidylinositol 4,5-bisphosphate-sensitive ion channels (Kir2.1) *J. Biol. Chem*. 2002 279: 47773 – 47782

Generally when studying an enhancement phenotype, it is best to start with conditions where the phenotype of the single mutant is weakest. Hence for the studies of double mutants of *rdgB*$^{9}$;*dEsyt*$^{KO}$, retinal degeneration in constant dark is shown in Fig 3A.

In studies where ER-PM CS is being observed and quantified, we have used constant dark since using light would trigger rhabdomere degeneration. Once that process has started, it is more difficult to observe and less instructive to comment on whether the ER-PM MCS is intact or not. Also as mentioned above even in the dark there is ongoing PLC activity and the disruption of ER-PM MCS of *dEsyt*$^{KO}$ in the dark is therefore rescued by genetically removing PLC activity (using the *norpA* mutant).

**Any explanation on why the RDGB staining is more punctate at day1 in *dEsyt*$^{KO}$ eyes (Fig. 4D)?**

***Response:*** The appearance of RDGB staining as punctate structures could be due to issues with antibody penetration. The image showing punctate staining is replaced with a better representative image where RDGB staining appears as crescent shaped structures at the base of the rhabdomere in *dEsyt*$^{KO}$ photoreceptors at day 1 post eclosion.

**The EM images are difficult to interpret. Why does the ER appear black in EM images? And why in some images does it have a bead-like appearance. Changes to the text and to the figure legends to better describe the EM micrographs will be helpful.**

***Response:*** We have adopted the osmium impregnation method (Suzuki E. et al., 1989, J. Neurocytology, Hotta lab) where samples were fixed in 2 % osmium for 4 days at 40$^0$C, leading to most endomembrane compartments being impregnated with osmium. Hence the ER appears black and is better visualized. This was important for our study as the SMC appear dark and thus provide very good contrast for visualization and counting. Bead like structures (perfectly spherical, black structures) are usually pigment granules present in red eyed flies that contribute to the red pigmentation of the fly eye. In all our EM experiments red eyed flies were used.

**Minor comments:**

**Further explanation of the *soD* mutation is warranted**

***Response:*** This has been rectified in the revised manuscript.

**Phalloidin staining of rhabdomeres in the Figure 4B WT condition appears diffuse. A better image would be desirable**

***Response:*** The phalloidin staining of the rhabdomeres appear diffuse since this is a section from the distal end of the photoreceptor (nearest to the lens) where the rhabdomeres are naturally close to each other with a small interommatidial space. We had to choose these sections since this was the location where the best dEsyt staining was seen rather than further down the photoreceptor where the intra-ommatidial space is larger and the rhabdomeres are much more distinct. The reason for this is as follows- these staining were done using whole mount immunohistochemistry followed by optical sectioning using confocal imaging. The dEsyt antibody (that recognizes the endogenous protein) is quite weak and we found it difficult to get good penetration into the full length of the retina and the best staining was always seen near the distal end of the photoreceptor. By contrast if one expresses a dEsyt::mCherry transgene and stains with the mCherry antibody one can get good images from any point along the length of the rhabdomere such as that shown in new Fig 4B.

**In the text Figure 4B is discussed prior to 4A. Please re-order either the text or the figure sequence.**

***Response:*** Figures reordered according to the text.

## 1st Revision - Editorial Decision 5th Jun 2020

Dear Dr. Padinjat,

Thank you for submitting the revised version of your manuscript. It has now been seen by one of the original referees and an external advisor.

As you can see, both the referee and the advisor find that the study is significantly improved during revision and recommend publication. Before I can accept the manuscript, I need you to address some minor points below:

• As per our guidelines, please add a 'Data Availability Section', where you state that no data were deposited in a public database.
• Please add 'Author Contributions' and 'Conflict of Interest' sections.
• Please convert the reference style to Harvard style. Please see https://www.embopress.org/page/journal/14693178/authorguide#referencesformat
• We noted that Appendix Fig S1 is currently not called out in the text.
• We realized that the EM images missing origin boxes for the magnifications.
• Papers published in EMBO Reports include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the key findings of the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb.
• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz Senyilmaz Tiebe

--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #2:

The authors have satisfactory addressed my first 2 comments. Concerning the third comment about the EM figures, I still do not fully understand those images. I find the fluorescence more compelling than the EM. It would be helpful to have a better labeling of the electron micrographs or new images.

External advisor:

I have had the chance to look at the revised manuscript from Padinjat, et al, as well as the reviewer comments. The reviewer concerns seem three-fold: 1) they felt that the current study lacked novelty due to similar reports in Change et al 2013, and
2) this present work may have been impacted by the publication of Hoffman et al, Dev Cell, which examines ER-PM contacts in yeast by Tricalbins (E-syt homologs).
3) there were minor technical concerns about the description of the observations in the Results section.

Based on my reading of the revised manuscript, I honestly feel that this revised work is generally well conducted and worthy of publication. This is for a few reasons:

1) While many of the key observations (eg. that loss of Drosophila E-Syt is essentially mild, and does not reduce ER-PM contacts in photo-receptor cells in early stages of Drosophila life), the fact that they observe a gradual age-dependent phenotype is pertinent to the field, and needs to be reported.

2) The work in general appears to be well conducted for a Drosophila-based study. The experiments are genrally well quantified, controlled, and generally conclusive.

Although the specific role of dESyt1 in dRGDb function is NOT fully elucidated here, this study provides important observations of E-Syt1 function in a model system (eg. Drosophila melaongraster) that is generally under-utilized in studies of inter-organelle membrane contact sites. Thus, given the overall quality of the work, and the fact that we are currently in a global pandemic that may preclude the completion of these studies in a timely manner, I honestly think the authors have adequately addressed the reviewer concerns.

## Editor Comments:

1. Add a 'Data Availability Section', where you state that no data were deposited in a public database

*Response: Added*

2. Add 'Author Contributions' and 'Conflict of Interest' sections

*Response: Added*

3. **Convert the reference style to Harvard style**

*This has been done.*

4. Appendix Fig S1 is currently not called out in the text

*Response: This has been rectified in the revised manuscript.*

5. EM images missing origin boxes for the magnifications

*Response: Rectified.*

6. Papers published in EMBO Reports include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the key findings of the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb.

*Response: Synopsis submitted as a word file*

7. Provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

*Response: subbitted as an image file*

8. Clarify several points in the figure legends (see attached document).

*Response: Rectified in the revised manuscript.*

**REFEREE #2**

1. **Better labeling of the electron micrographs or new images.**

   *This has been. Completed for Fig 5 and Fig 6*

## 2nd Revision - Editorial Decision 22nd Jun 2020

Prof. Raghu Padinjat
National Centre for Biological Sciences
Cellular Organization and Signalling
TIFR GKVK Campus
Bangalore, Karnataka 560065
India

Dear Prof. Padinjat,

Since my colleague Deniz Senyilmaz Tiebe is currently traveling, I stepped in as the secondary editor for your manuscript. I am very pleased to accept it for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Martina Rembold, PhD
Editor
EMBO reports

*****************************************************************************

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## Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

### A- Figures

### 1. Data

**The data shown in figures should satisfy the following conditions:**

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

### 2. Captions

**Each figure caption should contain the following information, for each panel where they are relevant:**

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi^2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

**In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.**

### B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

| Question | Answer |
|---|---|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | The sample size was chosen based on previous experience the variability seen in these types of experimental analysis. |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | For Drosophila studies, sample size was chosen based on prior experience and standards in the field. |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | For plotting the data, outliers were not excluded. |
| 3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe. | For all experiments, specific genotypes were generated using standard genetic crosses. Animals of the required genotype were allocated to specific experimental conditions at eclosion and followed as a function of age later in life. |
| For animal studies, include a statement about randomization even if no randomization was used. | All animals were collected after genotyping at eclosion and distributed randomly into groups that were subsequently subjected to distinct experimental conditions. |
| 4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe. | No |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | For experiements with Drosophila blinding was not done. |
| 5. For every figure, are statistical tests justified as appropriate? | Yes. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | Yes, the data meets the assumption of the test. One of the methods used in the statistical test is ANOVA, where it was used to do a compare analysis across grouped data sets. It takes into consideration the variations within a group as well as the variations between the groups for coming to a conclusive result. For example, if the variance between a group is much greater than the variations within a single group then that points to a situation where the means are not equal. |
| Is there an estimate of variation within each group of data? | Yes |

| | |
|---|---|
| Is the variance similar between the groups that are being statistically compared? | Yes |

**C- Reagents**

| | |
|---|---|
| 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right). | dEsyt antibody validation (Figure 4B and Appendix Figure S1 E); RDGB antibody validation (Yadav, S., Thakur, R., Georgiev, P., Deivasigamani, S., K, H., Ratnaparkhi, G., and Raghu, P. (2018). RDGBα localization and function at a membrane contact site is regulated by FFAT/VAP interactions. J. Cell Sci. 131, doi:10.1242/jcs.207985. - Fig 1A ii); antibodies: PLC, TRP, Gq and Rhodopsin (Balakrishnan, S.S., Basu, U., Shinde, D., Thakur, R., Jaiswal, M., and Raghu, P. (2018). Regulation of PI4P levels by PI4KIIIα during G-protein-coupled PLC signaling in Drosophila photoreceptors. J. Cell Sci. 131, jcs217257.- Fig 5) |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | NA |

* for all hyperlinks, please see the table at the top right of the document

**D- Animal Models**

| | |
|---|---|
| 8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals. | Drosophila strains were used in this study. Rearing conditions are described in the materials and methods section of the paper. |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | NA |
| 10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance. | NA |

**E- Human Subjects**

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | NA |
| 12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | NA |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | NA |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | NA |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | NA |
| 16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list. | NA |
| 17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines. | NA |

**F- Data Accessibility**

| | |
|---|---|
| 18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | NA |
| 19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right). | NA |
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**G- Dual use research of concern**

| | |
|---|---|
| 22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could. | NA |