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Goliath family E3-ligases regulate the recycling endosome pathway via VAMP3 ubiquitylation

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

Editor: Anke Sparmann

1st Editorial Decision 09 October 2012

Thank you for submitting your research manuscript (EMBOJ-2012-83097) to our editorial office. It has now been seen by three referees and their comments are provided below.

All reviewers appreciate your study and are in general supportive of publication in The EMBO Journal. Nevertheless, both referee #2 and #3 do express several points of criticisms that should be addressed based on the reviewer's constructive suggestions. Given the comments provided, I would like to invite you to submit a suitably revised manuscript to The EMBO Journal that attends to all raised concerns in full. I should add that it is our policy to allow only a single major round of revision and that it is therefore important to address the raised concerns at this stage. Please do not hesitate to contact me should any particular argument require further clarification.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

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

REFEREE COMMENTS

Referee #1

The manuscript describes the role of the goliath E3 ligases as key regulators of trafficking of internalized receptors back to the plasma membrane via recycling endosomes. Expression of RNF167, a human member of the Goliath E3-ligase family, ubiquitinates the SNARE protein, VAMP3, thus blocking recycling endosome trafficking. Furthermore, loss of VAMP3 reverses the Godzilla and RNF167 induced Rab5 positive giant endosome phenotype.
Overall, this is an original and comprehensive study that describes a novel mechanism that regulates sorting of cargoes in the endosomal compartment, in particular the recycling endosome trafficking via ubiquitylation of the VAMP3 SNARE protein. It is nicely written and well performed/controlled study.

Referee #2

The authors have clearly demonstrated that expression of the ubiquitin ligase, Godzilla (plus related ligases) induces the formation of enlarged early endosomes in a manner that requires ubiquitination of VAMP3. Interestingly it is not simply that ubquitination of VAMP3 inactivates the protein as the phenotype is not reproduced by depletion of VAMP3. The idea that SNARE function and, thereby, flux through a pathway can be modified by ubiquitylation of SNAREs is important and potentially of interest to readers of EMBO J. However I find the investigation into the effects of VAMP3 ubiquitylation on traffic rather superficial and these should, in my opinion, be expanded. In addition there are a number of figures that are described inadequately in the text and insufficient explanation is given. This particularly applies to effects on M6PR trafficking. Details are given below:

The overlap with EEA1 appears quite limited for Goliath, although is easier to see for Godzilla. This point is not made in the text. Figure 1C also shows costaining of Goliath and Godzilla with LC-3 which is a marker of autophagosomes. What is the significance of this?

Where is the reference to Fig 2A in the text? What is the significance of the M6PR staining? M6PR staining seems to disappear on Godzilla expression. What is the explanation for this?

The degree of endosomal enlargement seems pretty minor with Goliath (and the overlap with Rab5 is limited) and much more significant with Godzilla (as is the overlap with Rab5) - this should be pointed out in the text.

Fig 2C It would be helpful to see the GFP-Godzilla signal on its own as the degree of costaining with Rab 5 and FYVE looks limited.

The authors state that expression of wild type Godzilla abolishes the Rab 11 recycling endosome completely- it could just be that the distribution of the compartment has been disrupted such that it is no longer in a tight pericentriolar location.

The transferrin endocytosis experiments in Figure 7 D are important because they are the only direct measure of the role of ligase:Vamp3 interaction/ubiquitylation on traffic. Firstly, it appears that there is an inhibition of uptake of TF as there is no visible signal at 15 minutes whilst there is in the control. This is consistent with an inhibition of recycling as this would result in a depletion of TF receptor from the cell surface. Eventually some signal does appear in the enlarged endosomes but to conclude that it is prevented from recycling from this compartment it would be necessary to do some kind of ligand free chase for perhaps 1 hour (the chase could contain unlabeled TF) to show that TF is lost from the cell in controls and retained in the enlarged endosomes in the ligase-transfected cells.

Referee #3

This manuscript reports a novel role for the Drosophila Goliath E3-ligase and its substrate VAMP3/Syb in endocytotic cycling. The authors combine cell culture with fly genetics and used largely immunocytochemistry to localize endosomal markers following perturbation of goliath

levels (knockdown and overexpression). The important findings are the identification of VAMP3/Syb as a potential substrate of the E3 ligase and demonstration of ubiquitin-dependent roles of these proteins in endosomal trafficking. Overall, the data and images presented in the manuscript are of high quality. In fact, most of the figures are beautifully done! Ubiquitination plays important role in internalization of receptors and in ligand sorting following endocytosis. Hence, the key findings from this study are of general interest to readers in cell biology and development.

There are a number of issues that reduced my enthusiasm for this manuscript. Most of these concerns are relatively minor but the authors should address them.

1. Most of the colocalization of Goliath and Rab and other endosomal markers were conducted in cells or fly tissues in which Goliath or Godzilla was overexpressed. This is a concern because the authors have shown consistently that overexpression of Goliath or Godzilla induces the formation of large 'early' endosomes. In order to provide more convincing data on the localization of endogenous Goliath or Godzilla, the authors should show their colocalization or lack of colcoalization with endosomal markers (such as Rab5, Rab11, Rab7 etc) in cells as well as fly wing disc without overexpression. This can and should be easily done!

2.Figure 2: The overexpression phenotype for Godzilla and Goliath differs. This is consistent in both A and B panels. Do the authors have an explanation for this? In panel C, this experiment should be repeated using UAS-mCD8GFP to mark the cell membrane as it is unclear from the figure whether the formation of large Rab5+ vesicles is related to cell death. Alternatively, the authors should shown a DIC image to ensure that the cell polarity and integrity remains intact.

3.The authors observed similar enlargement of early endosomes in Goliath LOF and GOF, but failed to explain why. In the working model,if ubiquitination of VAMP3 is important for endosomal trafficking, how can LOF and GOF fit into the model? If LOF mimics VAMP3 K66, 68, and 77R mutant, why would GOF produce a similar phenotype? Please address this either experimentally or theoretically so that readers can follow the logic of these phenotypes.

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6. While most experiments were done with RNAi or overexpression, mutant clones were not efficiently used (contrary to most fly folks). It is unclear why the authors examined JNK signaling in the wing disc. How about other signaling pathways such as Wing itself or Delta-Notch? Furthermore, is the down regulation of JNK signaling related to failure in internalization of receptors or recycling? In both cases, would you predict an upregulation of signaling as the authors stated that signaling can be amplified on the plasma membrane as well as in endosomes? In other words, the functional aspect of goliath analysis was not done well (and the text was rushed on this part too).

7.The Tfn data do not convince this reviewer whether the primary defect occurs in internalization of receptors, degradation of internalized receptors by lysosomes, or recycling itself. The authors seemed to prefer the recycling route, but the evidence is weak. Can the authors provide stronger evidence to distinguish an internalization defect vs recycling defect?

1st Revision - authors' response 30 November 2012

The manuscript describes the role of the goliath E3 ligases as key regulators of trafficking of internalized receptors back to the plasma membrane via recycling endosomes. Expression of RNF167, a human member of the Goliath E3-ligase family, ubiquitinates the SNARE protein, VAMP3, thus blocking recycling endosome trafficking. Furthermore, loss of VAMP3 reverses the Godzilla and RNF167 induced Rab5 positive giant endosome phenotype.
Overall, this is an original and comprehensive study that describes a novel mechanism that regulates sorting of cargoes in the endosomal compartment, in particular the recycling endosome trafficking via ubiquitylation of the VAMP3 SNARE protein. It is nicely written and well performed/controlled study.

We thank reviewer 1 for their kind and generous remarks regarding our work.

Referee #2

The authors have clearly demonstrated that expression of the ubiquitin ligase, Godzilla (plus related ligases) induces the formation of enlarged early endosomes in a manner that requires ubiquitination of VAMP3. Interestingly it is not simply that ubquitination of VAMP3 inactivates the protein as the phenotype is not reproduced by depletion of VAMP3. The idea that SNARE function and, thereby, flux through a pathway can be modified by ubiquitylation of SNAREs is important and potentially of interest to readers of EMBO J. However I find the investigation into the effects of VAMP3 ubiquitylation on traffic rather superficial and these should, in my opinion, be expanded. In addition there are a number of figures that are described inadequately in the text and insufficient explanation is given. This particularly applies to effects on M6PR trafficking. Details are given below:

1. The overlap with EEA1 appears quite limited for Goliath, although is easier to see for Godzilla. This point is not made in the text.

We agree with the reviewer that the EEA1 overlap with Godzilla is easier to see than that of Goliath. However, a large amount of Goliath does still overlap with Goliath, even though it may not be as easy to see as for Godzillla. To clarify this and address the reviewers comments we have amended the text on Page 8 to: *A significant amount of Godzilla clearly colocalises with endosomal markers, such as EEA1, upon overexpression (Figure 1D). While less extensive than that observed with Godzilla, overlap with EEA1 is also observed with the related Goliath (Figure 1D and 2A).*

Figure 1C also shows costaining of Goliath and Godzilla with LC-3 which is a marker of autophagosomes. What is the significance of this?

A close relationship has been described between between autophagy and endocytosis with both sharing lysosomes as their common end-point. It has been shown that autophagy requires a functional endocytic pathway. Given that autophagy shares its trafficking pathway with endocytic trafficking, it is possible that autophagocytosis mediated trafficking might also be affected by Godzilla, either directly or indirectly. Autophagosomes are described as fusing with endosomes (Huotari, EMBO J, 2011). With our present knowledge we interpret the overlap with LC3 we as autophagosomes fusing into the enlarged endosomes formed upon expression of Godzilla and Goliath.

2. Where is the reference to Fig 2A in the text? What is the significance of the M6PR staining? M6PR staining seems to disappear on Godzilla expression. What is the explanation for this?

In our initial attempts to understand the role of Godzilla we also noted the loss of M6PR staining which we interpreted at that point to be a block in the endosomal maturation process. In spite of testing all available published Rab5 and Rab7 regulators as targets of Godzilla, we were unable to identify any Godzilla targets. Our interpretation of this now is that endosome maturation may be affected on Godzilla expression and that this is why we see a lack of M6PR staining. We have now included the following text in the revised manuscript to address both this comment and to include description of Figure 2A, which we had somehow missed describing in the initial version: *'A significant amount of Godzilla clearly colocalises with endosomal markers, such as EEA1, upon overexpression (Figure 1D). While less extensive than that observed with Godzilla, overlap with EEA1 is also observed with the related Goliath (Figure 1D and 2A). In general we noted that*

expression of Godzilla results in a stronger enlarged endosome phenotype than Goliath. This was also reflected in the more obvious loss of Mannose-6-Phosphate Receptor staining in cells expressing Godzilla, suggesting that Godzilla expression may lead to perturbations in the endosomal maturation process (Figure 2A).'

3. The degree of endosomal enlargement seems pretty minor with Goliath (and the overlap with Rab5 is limited) and much more significant with Godzilla (as is the overlap with Rab5) - this should be pointed out in the text.

Both reviewer 2 and 3 have noted that Goliath is not as potent as Godzilla in the production of large endosomes. This is indeed the case and we have now pointed this out in the text on Page 8 of the manuscript with the following text: *'Consistently, we noted that Godzilla was significantly more potent in the production of dramatically enlarged endosomes than Goliath'.* While overall Godzilla is more potent with almost every cell developing very large endosomes, there is a range of endosome sizes observed (in our quantifications we use a cut-off of 3mm for enlarged endosomes). It is possible that there is a difference in the substrate specificity of Godzilla and Goliath, or in an important protein interaction, or in fact many possible scenarios that may explain their slightly different potencies. At the present time we cannot provide an answer to this, however, it is important to note that in some cells Goliath produces a very strong phenotype, comparable to Godzilla (seen for example in Panel D of Figure 1). In general, in Goliath expressed cells the endosomes are large; it is simply that in Godzilla expressing cells they often become huge.

4. Fig 2C It would be helpful to see the GFP-Godzilla signal on its own as the degree of costaining with Rab 5 and FYVE looks limited.

Both reviewer 2 and 3 have raised issues with Figure 2C. While we have unfortunately been unable to use UAS-mCD8GFP as was suggested by reviewer 3 for this analysis since Godzilla is also GFP-tagged, we have redone this Figure to address both reviewers' comments. The strongest data is clearly from the lower panel in which mCherryFYVE is seen on enlarged endosomes. These mCherryFYVE positive enlarged endosomes also contain Godzilla-GFP in some subdomains of the endosomes. We have also included an additional analysis with phalloidin as a cell defining marker which is now included as Figure S4 in the revised version.

5. The authors state that expression of wild type Godzilla abolishes the Rab 11 recycling endosome completely- it could just be that the distribution of the compartment has been disrupted such that it is no longer in a tight pericentriolar location.

The reviewer does have a point. In fact, on Godzilla overexpression we can see some residual Rab11 staining which overlaps with Godzilla, and is therefore not in its normal pattern of distribution. At the present time we consider this may reflect the lack of generation of Rab11 containing recycling endosomes. We feel our data, including the Transferrin chasing experiments, supports the fact that recycling is no longer functioning. However, we have amended this sentence with a better description of this data (at the bottom of Page 12) and to address the point raised by the reviewers comment.

6. The transferrin endocytosis experiments in Figure 7 D are important because they are the only direct measure of the role of ligase:Vamp3 interaction/ubiquitylation on traffic. Firstly, it appears that there is an inhibition of uptake of TF as there is no visible signal at 15 minutes whilst there is in the control. This is consistent with an inhibition of recycling as this would result in a depletion of TF receptor from the cell surface. Eventually some signal does appear in the enlarged endosomes but to conclude that it is prevented from recycling from this compartment it would be necessary to do some kind of ligand free chase for perhaps 1 hour (the chase could contain unlabeled TF) to show that TF is lost from the cell in controls and retained in the enlarged endosomes in the ligase-transfected cells.

We thank both reviewer 2 and 3 for their comments on this experiment and the results. We have now addressed this and strengthened this data by carrying out the chase experiments as suggested by reviewer 2. The results are in agreement with inhibition of recycling and retention of transferrin in the enlarged endosomes in the Godzilla or RNF167 transfected cells. This data is now included as Figure 8 with according alterations in the text in the revised version of the manuscript.

Referee #3

This manuscript reports a novel role for the Drosophila Goliath E3-ligase and its substrate VAMP3/Syb in endocytotic cycling. The authors combine cell culture with fly genetics and used largely immunocytochemistry to localize endosomal markers following perturbation of goliath levels (knockdown and overexpression). The important findings are the identification of VAMP3/Syb as a potential substrate of the E3 ligase and demonstration of ubiquitin-dependent roles of these proteins in endosomal trafficking. Overall, the data and images presented in the manuscript are of high quality. In fact, most of the figures are beautifully done! Ubiquitination plays important role in internalization of receptors and in ligand sorting following endocytosis. Hence, the key findings from this study are of general interest to readers in cell biology and development.

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In order to address the reviewer's comments we have performed a number of experiments. These are the localisation of endogenous Godzilla with GFP-tagged endosome markers such as Rab5, Rab7, or Rab11 which have been expressed in *Drosophila* tissues. The data shown in this revised version is via expression in the salivary gland (using the AB1-Gal4 driver) as well as in the wing disc using the MS1096-Gal4 driver. This data is now included as Figure S1E and F. We see colocalisation of endogenous Godzilla with both Rab5 and Rab7 endocytic compartments in this analysis. This analysis is backed up by use of antibodies to endogenous Rab5,7 and 11 in Figure S1G. We feel that the strongest and clearest data supporting the presence of Godzilla in the endosomal compartment comes from the genetic trick of overexpressing activated Rab5 - Rab5Q88LYFP (Stenmark et al., 1994). This results in the production of enlarged endosomes – which are highly decorated with endogenous Godzilla protein (Figure 1E). In this case all visible endogenous cellular Godzilla is accumulated on the activated Rab5 induced enlarged endosomes. We have altered the text on Page 8/9 to include this new data.

2. Figure 2: The overexpression phenotype for Godzilla and Goliath differs. This is consistent in both A and B panels. Do the authors have an explanation for this?

Both reviewer 2 and 3 have noted that Goliath is not as potent as Godzilla in the production of large endosomes. This is indeed the case and we have now pointed this out in the text on Page 9 of the manuscript with the following text: *'Consistently, we noted that Godzilla was significantly more potent in the production of dramatically enlarged endosomes than Goliath'.* While overall Godzilla is more potent with almost every cell developing very large endosomes, there is a range of endosome sizes observed (in our quantifications we use a cutoff of 3mm for enlarged endosomes). It is possible that there is a difference in the substrate specificity of Godzilla and Goliath, or in an important protein interaction, or in fact many possible scenarios that may explain their slightly different potencies. At the present time we cannot provide an answer to this, however, it is important to note that in some cells Goliath produces a very strong phenotype, comparable to Godzilla (seen for example in Panel D of Figure 1). In general, in Goliath expressed cells the endosomes are large; it is simply that in Godzilla expressing cells they often become huge.

In panel C, this experiment should be repeated using UAS-mCD8GFP to mark the cell membrane as it is unclear from the figure whether the formation of large Rab5+ vesicles is related to cell death. Alternatively, the authors should shown a DIC image to ensure that the cell polarity and integrity remains intact.

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Our current thinking is that Godzilla may function as a molecular switch of early to recycling endosome to regulate cargo trafficking at the early endosome. Although we do not have direct evidence of what effect ubiquitylation may have on VAMP3 structure and SNARE complex formation, we assume that the zipping process of VAMP3 with its partner SNARE proteins would be affected by the ubiquitylation around the last layer of the SNARE domain (Hernandez et al., 2012). According to this hypothesis the large endosome phenotype in both Godzilla GOF and LOF could be caused by continuous receptor-mediated endocytosis in the cells in which the endosome recycling process is impaired. At the current time we have little knowledge of the effect of ubiquitylation, for example on SNARE complex formation either specially or temporally. To respond to this comment, we have amended the text in the results and discussion.

4. Based on the data provided in the manuscript this reviewer failed to understand why Lys 66, 68, and 77 were shown to be the target of the E3 ligase.

The proteomics based analysis which we employed in this study is able to identify not only the protein substrate of the ligase, but also the individual lysine residues that are ubiquitylated within the substrate. This is done by means of an antibody specific to conjugated form of ubiquitin followed proteolysis and mass spectrometry analysis on both Godzilla and control samples. This analysis lead to the identification of the individual lysines within VAMP3 that are ubiquitylated by Godzilla. To clarify this for the reader, we have included a sentence within the text on page 11.

5. The most interesting and surprising data are that reduction of VAMP3 abolished the formation of large early endosomes and further 'rescued' endosomal trafficking. How can these be explained? Does syb mutation 'rescue' the large endosome in wing discs? Does VAMP3 or syb LOF rescue goliath-induced transferrin receptor recycling? The loss of giant endosomes can be explained by the reduction of VAMP3, but how does this rescue the subsequently event of Rab-11 dependent recycling? Are Rab11+ vesicles restormed in VAMP3 RNAi Godzilla-overexpressing cells?

The reviewer also asks whether loss of Syb affects the large endosome phenotype in wing discs. Indeed, we find that loss of Syb does have a similar effect in discs. These results are included as Figure 5G + H of the revised version of the manuscript, where we see a significant rescue of the Rab5 positive endosomes upon loss of Syb in wing discs, as induced by RNAi (UAS-SybRNAi). While VAMP3 reduction by its own does not cause the enlargement of EEA1 endosome (Figure S8), suggesting active recycling, it appears that VAMP3/Syb plays an important role in the modulation of endosomal trafficking by Godzilla. Our data also suggests that the giant endosome induced by Godzilla is not mediated by a simple inhibition of VAMP3 by ubiquitylation, and that perhaps both ubiquitylation and deubiquitylation may play important roles in this process.
In response the reviewers question concerning whether Rab11+ vesicles are restored in VAMP3 RNAi Godzilla-overexpressing cells we have performed this experiment. We find that upon VAMP3 RNAi Godzilla-overexpressing cells we observe some punctate Rab11 structures within HEK293 cells. However, they do not resemble a normal Rab11 recycling pattern at all, and we are unsure of their identity or significance. Since we do not see a convincing restoration of the Rab11 recycling vesicle pattern we have not included this data in the revised version.

6. While most experiments were done with RNAi or overexpression, mutant clones were not efficiently used (contrary to most fly folks). It is unclear why the authors examined JNK signaling in the wing disc. How about other signaling pathways such as Wing itself or Delta-Notch?

Furthermore, is the down regulation of JNK signaling related to failure in internalization of receptors or recycling? In both cases, would you predict an upregulation of signaling as the authors stated that signaling can be amplified on the plasma membrane as well as in endosomes? In other words, the functional aspect of goliath analysis was not done well (and the text was rushed on this part too).

The reviewers point here is well taken. We have focused strongly on trying to understand how the Godzilla protein may be working, but have not had time to complete the fly analysis *in vivo* in terms of signalling to a high level. While we can show there are effects on signalling *in vivo* in the fly, which we tried to convey with the JNK signalling result, we have not had time to tease this apart in the fly. On consideration, given the reviewers point that this is not well done (or perhaps not completely done), and that the data does not significantly add anything to the manuscript, we have removed this from the manuscript and will work on a more complete clonal analysis of various signalling processes in the future.

7. The Tfn data do not convince this reviewer whether the primary defect occurs in internalization of receptors, degradation of internalized receptors by lysosomes, or recycling itself. The authors seemed to prefer the recycling route, but the evidence is weak. Can the authors provide stronger evidence to distinguish an internalization defect vs recycling defect?

We thank both reviewer 2 and 3 for their comments on this experiment and the results. We have now addressed this and strengthened this data by carrying out the chase experiments as suggested by reviewer 2. The results are in agreement with inhibition of recycling and retention of transferrin in the enlarged endosomes in the Gozdilla or RNF167 transfected cells. This data is now included as Figure 8 with according alterations in the text in the revised version of the manuscript.

2nd Editorial Decision 02 January 2013

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal. In order to proceed with the production process, we will require some additional information.

- Please indicate the number of biological replicates, which the photomicrographs represent in the figure legends of Figures 1, 2, 4 and 8. To simplify the processing, you can just sent the amended text file in reply to this e-mail.

- Please complete and sign the linked license agreements (see below).

If you have any questions, please do not hesitate to contact me directly.

Thank you for your contribution to The EMBO Journal and my best wishes for a happy and successful New Year!

REFEREE COMMENTS

Referee #3

The authors did a very good job addressing my previous comments and the comments of other reviewer. I believe this revised version is of high quality and should be of general interest to readers of EMBO Journal.

