THE EMBO JOURNAL

Manuscript EMBO-2011-77041

A Drosophila Metallophosphoesterase Mediates Deglycosylation of Rhodopsin

Jinguo Cao, Yi Li, Wenjing Xia, Keith Reddig, Wen Hu, Wei Xie, Hong-Sheng Li and Junhai Han

Corresponding author: Junhai Han, Southeast University,

Review timeline:

Submission date:	19 July 2010
Editorial Decision:	23 July 2010
Editorial Decision:	04 March 2011
Revision received:	02 June 2011
Editorial Decision:	28 June 2011
Revision received:	30 June 2011
Accepted:	01 July 2011

Transaction Report:

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

1st Editorial Decision 23 July 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. I have now had a chance to read it carefully and to discuss it with my colleagues and I am sorry to say that we cannot offer to publish it.

We appreciate that you have provided evidence that the metallophosphoesterase dMPPE is involved in the deglycosylation of rhodopsin, and that this impacts on rhodopsin trafficking to/stability at the rhabdomere. We do recognise that this provides insight into the relevance of Rhodopsin deglycosylation (although we note that some of the phenotypes appear rather weak), and also that it delineates an in vivo role for this enzyme. However, our major concern here is that it remains completely unclear how dMPPE might mediate deglycosylation, or how direct this effect might be. In the absence of clear mechanistic insight, I am afraid we find that your study remains rather preliminary. Moreover, it is not clear whether this represents a very specific effect of dMPPE on Rh1, or whether dMPPE (and/or its homologs) might be more generally involved in regulating glycosylation status of transmembrane proteins. Given these concerns, I am sorry to say that we do not feel that your manuscript is well suited to publication in the EMBO Journal, and we can not offer to consider it further.

Please note that we publish only a small percentage of the many manuscripts that we receive at the EMBO Journal, and that the editors have been instructed to subject only those manuscripts to external review which are likely to receive enthusiastic responses from our reviewers and readers. I am sorry to have to disappoint you on this occasion, but I hope that this negative decision will not prevent you from considering the EMBO Journal for publication of future studies.

Yours sincerely,

Editor
The EMBO Journal

2nd Editorial Decision 04 March 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first apologise for the delay in getting back to you with a decision: as I told you, we experienced some difficulty in finding three appropriate reviewers, and have only just received the third report. The comments of all three referes are enclosed below. As you will see, all three referees express interest in your work, but raise a number of concerns that would need to be addressed before we could consider publication.

To outline these briefly:
- Referees 1 and 3 both comment that evidence for a causal link between dMPPE, alpha-Man-II and Rh1 deglycosylation is still missing. To this end, it would be essential to show that the reason for the dMPPE mutant phenotype is hyper-phosphorylation of alpha-Man-II. Both referees suggest that identifying the phosphorylation site(s) on alpha-Man-II and generating phosphomutants would be the way to demonstrate causality here, and we such experiments would be critical for an eventual positive outcome here.
- All three referees raise a number of technical concerns - particularly with quantifications and image quality - that need to be dealth with.
- I would encourage you to follow the referees' recommendation to improve the writing throughout, and suggest that professional proofreading, or having your manuscript seen by a native-speaking colleague, would be very valuable here.
- Referee 3 argues that you have not demonstrated how alpha-Man-II activity actually induces complete Rh1 deglycosylation. We take his/her point, and should you have any further insight into this, it would clearly be valuable. However, we would not see a complete elucidation of the mechanism of Rh1 deglycosylation downstream of alpha-Man-II as being essential for publication here.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. 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 initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on 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. Also, please don't hesitate to get in touch if you have any questions or comments regarding the revision.

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

Best wishes,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors describe the phenotype of a mutant of CG8889, a metallophosphoesterase referred to as dMPPE, on the maturation of Rhodopsin1 in Drosophila retina. They show that in dMPPE mutant, Rhodopsin1 has a higher molecular weight due to glycosylation suggesting an altered deglycosylation during Rhodopsin1 maturation. Glycosylated Rhodopsin1 still reaches the rhabdomeres, mediates phototransduction and is endocytosed at a similar rate but its transport to the rhabdomere is slowed down and once endocytosed it is less stable. Authors show that dMPPE has a phosphoesterase activity and no deglycosidase activity as expected. They check that ectopic expression of dMPPE rescues Rhodopsin1 maturation. They identify hyperphosphorylated proteins in mutant tissue, especially α-Man-II, a glycosidase. They show that dMPPE and α-Man-II colocalize partially, interact and that dMPPE regulate the phosphorylation of α-Man-II. Mutant for α-Man-II exhibits also a similar rhodopsin1 deglycosylation defect. Finally, in vitro, α-Man-II is able to partially deglycosylate the glycosylated rhodopsin1 from the dMPPE mutant.

Overall this is an interesting story with a large body of work. It is quiet novel because the physiological significance of Rh1 deglycosylation is not clear.
Experiments well conceived and results of good quality. However the general presentation needs to be improved. I suggest also some experiments that would clarify some aspects of the paper. The authors should carefully address the following points:

Specific points:

In the introduction or in the discussion, the authors should stress the difference between their mutant and classical Rh1 maturation mutant such as ninaA. While in ninaA mutation lead to misfolded Rh1 accumulation in the ER and ER stress (Colley et al. 1991; Mendes et al. 2009), dMPPE does not block ER exit but lead to trafficking problems.

The paper requires a careful proofreading. English should be improved, abbreviations explained and figure numbering in the text should correspond to the right figure!
For example:
P4: MPPEs may hydrolyze a wide variety of protein and nucleotide substrates, such as protein phosphoserine phosphatases, sphingomyelin phosphodiesterases, nucleotidases, 2',3'-cAMP phosphodiesterases, and nucleases.
P5: on GPI-APs by removal of a side-chain EtNP from Man2 of GPI
P5: cross-eye sections
P8: Fig3B, Fig3C
P12: transporting
P14: it is a high chance that
P15: Fig7G, Fig7H
P16: Our study has revealed the physiological importance of deglycosylation of Rh1 rhodopsin, which is for its rapid transportation and stability, but is not for its rhabdomeral targeting and signaling function.
P17: However, dMPPE can not remove the oligosaccharide chain from glycosylated Rh1, it is a phosphatase. It seems there is a gap between the dephosphorylation function of dMPPE and Rh1 deglycosylation defects. α-Man-II might filled in this gap.
P17: phosphorylation modification in serine site
P18: which in tune was
P19: these two proteins show the similar metal-dependent enzyme activation manner
In figure legends and material method: SEMs
P25: Figure 3 C
P26: bisected

- figure legends need to be written again. They are usually not describing the results enough and what has been done. Some are really confusing, e.g. the title of Fig1E is "reduction of Rh1 protein level in newly eclosed dmppe mutants" whereas the opposite in written in the text. The title of Fig3 C mention dmppe mutant instead of wild-type.

- About figures, scale bars are usually missing, molecular weights for western blot as well (Rh1 monomer or dimer). Graph y axis is usually deformed. Western blots are usually much too saturated

to allow quantification. Images do not have enough definition.
- p6: "Rh1-containing large vesicle appeared to be the aggregation of small vesicle (Fig 1D). This is not clear on the figure and what does it implied?

- Fig3E: % of signal in rhabdomere not in cytoplasm.

- Fig3CDE: results redundant with Fig1C

- p9: "In two-day-old dark-reared flies, dmppe mutants displayed normal ERG responses (Fig S1 A), with the sensitivity to light at the wild-type level (Fig S1 B)." Quantification of the plateau and the prolonged depolarization afterpotential (PDA) is necessary to conclude that rh1 has a normal behavior in ERG. Quantification of ON and OFF transients would complete the ERG analysis

- Fig4: It is not demonstrated that the decrease light sensitivity of young and old mutant flies, the decrease expression of Rh1 in old mutant flies and the altered rhabdomere structures of mutant flies are due to the defect of rh1 maturation. One could imagine that dMPPE has other target than α-Man-II and the maturation of Rh1. The decrease of INAD in 20-day-old flies is not commented.

- Many panels of Fig1 and Fig4 do not correspond to the title of the figures.

-p11: Informations about lethality and nullity of the mutation should be added

-Fig5C, 5D : immunoblotting of dMPPE is necessary as a control of the ectopic expression of dMPPE.

- Staining in Fig6A can be improved (especially rh1), and a DAPI staining would be appreciated

- Fig6D: a costaining to orient the structure is required. Are the rh1 and MPPE staining localized in the forming rhabdomers?

- Fig S3 : western blots are not convincing

- Fig7 : numerous results correlate with α-Man-II as the mediator of dMPPE for rh1 deglycosylation. Rescuing rh1 deglycosylation in dMPPE mutant with a constitutively active form of α-Man-II (such as a non phosphorylable form) would prove that α-Man-II is the mediator of dMPPE for rh1 deglycosylation.

- p16: generating the double mutant α-Man-II/edem1, edem2, png1, α-Man-I, α-Man-IIb and CG14015 are more prone to identify the glycosidase that further remove the remaining GnMan3Gn2 yielded by α-Man-II hydrolysis.

-p19: "it renders Rh1 more sensitive to endocytic degradation, and could cause morphological and functional defects in the photoreceptor cells of old dmppe flies." This hypothesis does not correspond to the conclusion of a recent paper Chinchore et al 2009 which describes that accumulation of rhodopsine1 in endocytic compartment, due to an absence of degradation for exemple, elicit cell death of the photoreceptor

Referee #2 (Remarks to the Author):

This is an interesting manuscript which shows for the first time that a phosphoesterase, dMPPE mediates the deglycosylation of membrane receptor by regulating the phosphorylation/dephosphorylation status of a-ManII. The evidences they show here, Rh1 deglycosylation requires dMPPE are quite solid, and the Man-II involvement also seems to be true. Regarding my knowledge, this is a novel finding, and interesting issue for broad audience.

Moreover, for visual science field people, the two facts would be quite surprising and important: Rh1 deglycosylation is not important for 1) its rhabdomere targeting nor 2) its signaling function. Rh1 glycosylation and deglycosylation is found long time ago, and the importance of the

glycosylation were investigated, but not for deglycosylation.

I highly recommend this paper published in EMBO Journal, but they should address some specific points.

1) Regarding Fig 1C, I wonder how quickly authors fixed flies after they moved dark reared flies to light condition (I guess they fixed flies with light). As within 20mins after illumination, many RPPs are formed, 10-20mins difference for fixation speed gives a big difference in RPPs number. In other words, it there really many RPPs in complete dark reared dMPPE mutant?

3) There is no information about anti Man II antibody.

4) Regarding dMPPE localization. They showed partial colocalization of dMPPE with calnexin and Syntaxin6 in dMPPE tranfected HEK293 and stated dMPPE predominantly exists in ER and partial resides in Golgi. However, Fig 6B and D show endogenous dMPPE localizes on Golgi bodies in fly photoreceptor. I guess over expression study often overflows proteins from its real destination. I think they can say more strongly dMPPE localization on Golgi in fly photoreceptors.

5) Why does dMPPE mutant photoreceptor degenerate? Is this because of quick Rh1 degradation in dMPPE mutant? Is this light dependent degeneration ?

6) Typo P8 middle (Fig3C ->3B)s

Referee #3 (Remarks to the Author):

This manuscript describes the impact of a loss-of-function mutation in a Drosophila metallophosphoesterase on the processing and stability of rhodopsin (Rh1). It has been previously shown that Rh1 is post-translationally modified with a single N-linked glycan that is removed as the protein matures through the secretory pathway. Here the authors propose that this glycan must be processed by Golgi-a-mannosidase II before complete removal and that activation of Mannosidase II requires dephosphorylation by a metallophosphoesterase (dMPPE). The authors demonstrate that maturation and rhabdomere targeting of Rh1 is only mildly affected in the mutant. Likewise, Rh responses to light show only a small decrement in the mutant. However, long-term stability of Rh1 in the light adapted eye is decreased, perhaps due to increased endocytosis. In general, the manuscript presents a reasonable characterization of dMPPE and the minor changes in function associated with its decreased activity. The most exciting finding in this paper is the observation that Mannosidase II activity is required for deglycosylation of Rh1 and that this function of Mannosidase II may be modulated by direct phosphorylation. However, aspects of the data that are meant to support this model are not completely developed and key components of the mechanism leading to deglycosylation remain unidentified, making it difficult to assess the relative importance of Mannosidase phosphorylation. Furthermore, many of the experimental details are difficult to decipher and the manuscript contains a large number of formatting, spelling, and grammatical errors.

Major Comments:

1. Figure 7, panels F-H. These data are the key findings related to processing of Rh1. First of all, panels G and H are mislabeled in the legend or figure. But, regardless, the following is clear, and very interesting: Rh1 fails to be processed in the Man-II mutant just as it remains unprocessed in the mppe mutant. This result indicates that Man-II is necessary for deglycosylation of Rh1. However, it does not necessarily mean that Mppe is necessary for Man-II activation. In fact, the panel labeled "G" in Figure 7 suggests otherwise. The last two lanes of this panel show that Man-II reduces the molecular weight of Rh1 to the same extent, with or without MPPE. It is very difficult, however, to figure out what was actually done in panel G. The methods section does not describe the experiment and the figure legend is extremely minimal. I'm assuming that Rh1 from mppe mutant was treated with some form of recombinant MPPE and Man-II in vitro, not in vivo. If Man-II requires MPPE activation, why is it active on Rh1 in vitro? The result in Panel H is also confusing. Man-II should reduce the glycan to a smaller core than the 2,3-Mannosidase, yet incubation with the 2,3-Mannosidase almost reduces the mass all the way to the deglycosylated form. Can the authors

propose a glycan structure or structures that might satisfy the data? Also, essentially no details are given for how the exoglycosidase treatments were done.

2. A major piece of the mechanism is missing. How is the glycan removed following Man-II action? The authors state that Drosophila PNGase mutants (png) are not deficient in deglycosylation, so that would tend to rule out an ERAD-type mechanism. Without proposing or identifying a mechanism for deglycosylation, it remains unclear how Man-II regulation is relevant for the deglycosylation process and the data becomes more of a preliminary report than a definitive finding.

3. The proteomic data presentation is insufficient. Is it true that peptides belonging to no other proteins were found in the gel slices at 130 and 51 kD? What is the percent coverage for Man-II? What is the confidence level for the protein id? As presented, it seems that Man-II may have been pursued because of it's role in glycan processing rather than because of the strength of the proteomic id. The quality of the anti-phosphopeptide antibody blots in supplement Fig. S3 does not clearly support that bands at 130 and 51kD are of interest. So, one has to assume that the selection of these targets was based on the silver stain (is it a silver stain?) in panel A of figure 7. The id of these proteins as phosphoproteins is based solely on metal ion affinity chromatography, which can also enrich for sulfated, sialylated, or mannose-phosphorylated proteins. Therefore, without actually mapping a phosphorylation site on the targets, it is not clear how to test whether phosphorylation detected in vitro is functionally relevant in vivo.

Minor Comments:

1. Fig. 1, Panel B: It is not clear from the methods section what is meant by "Rela light sensitivity (%)." Is this based on response at a single light intensity or averaged over many intensities? If so, what intensity or intensities were chosen? Panel C: Do the authors mean "RPV" or "RPP" for the y-axis? Panel D: The authors should provide more context for the EM. The significance of the inset is not clear.

2. Fig. 3, Panel A,B: Something is mislabeled here. Panel E: Shouldn't the y-axis be "% of signal in Rhabdomere" not "Cytop."

3. Fig. 5, Panel E: Again, shouldn't "RPP" be "RPV?"

4. Supplement Fig. 2 is not necessary.

5. Supplement Fig. 3: What are the arrows meant to indicate?

6. Numerous instances of improper words are found throughout the manuscript. A rigorous proof-reading by a native English speaker would help the readability of the manuscript.

1st Revision - authors' response 02 June 2011

Referee #1:

Specific points:

In the introduction or in the discussion, the authors should stress the difference between their mutant and classical Rh1 maturation mutant such as ninaA. While in ninaA mutation lead to misfolded Rh1 accumulation in the ER and ER stress (Colley et al. 1991; Mendes et al. 2009), dMPPE does not block ER exit but lead to trafficking problems.
In the new version, we have stressed the difference between *dmppe* mutant and classical Rh1 maturation mutant such as *ninaA* in the discussion, and have cited the relevant literatures.

The paper requires a careful proofreading. English should be improved, abbreviations explained and figure numbering in the text should correspond to the right figure!
For example:
P4: MPPEs may hydrolyze a wide variety of protein and nucleotide substrates, such as protein phosphoserine phosphatases, sphingomyelin phosphodiesterases, nucleotidases, 2',3'-cAMP

phosphodiesterases, and nucleases.
P5: on GPI-APs by removal of a side-chain EtNP from Man2 of GPI
P5: cross-eye sections
P8: Fig3B, Fig3C
P12: transporting
P14: it is a high chance that
P15: Fig7G, Fig7H
P16: Our study has revealed the physiological importance of deglycosylation of Rh1 rhodopsin, which is for its rapid transportation and stability, but is not for its rhabdomeral targeting and signaling function.
P17: However, dMPPE can not remove the oligosaccharide chain from glycosylated Rh1, it is a phosphatase. It seems there is a gap between the dephosphorylation function of dMPPE and Rh1 deglycosylation defects. α-Man-II might filled in this gap.
P17: phosphorylation modification in serine site
P18: which in tune was
P19: these two proteins show the similar metal-dependent enzyme activation manner
In figure legends and material method: SEMs
P25: Figure 3 C
P26: bisected
We thank the reviewer for pointing out those mistakes and English problems. We have corrected the figure numbers, defined the abbreviations, and had a native English speaker improve the writings. A single exception is the word *bisected*, which is the one we intended to use and thus has not been changed in the manuscript.

- figure legends need to be written again. They are usually not describing the results enough and what has been done. Some are really confusing, e.g. the title of Fig1E is "reduction of Rh1 protein level in newly eclosed dmppe mutants" whereas the opposite in written in the text. The title of Fig3 C mention dmppe mutant instead of wild-type.
We have rewritten figure legends to correct those mistakes and to include more details.

- About figures, scale bars are usually missing, molecular weights for western blot as well (Rh1 monomer or dimer). Graph y axis is usually deformed. Western blots are usually much too saturated to allow quantification. Images do not have enough definition.
In the new version, we have added scale bars in all image data and marked molecular weights in western blots. We have corrected the deformation of graphs. We have also replaced those saturated western blots data with shorter exposed ones, and have improved the quantity of images, such as Figure 1D.

- p6: "Rh1-containing large vesicle appeared to be the aggregation of small vesicle (Fig 1D). This is not clear on the figure and what does it implied?
In the new version, we have improved the image quantity in Figure 1D. In the Results part, we have reworded the sentence as "…RPVs appeared to be collections of small vesicles, which are reminiscent of the reported MVBs". Since the reported MVBs mediate trafficking of newly synthesized Rh1 protein, the data suggests that Rh1 could be retained in the secretion pathway in the *dmppe* mutant. This implication has been made clear in the text.

- Fig3E: % of signal in rhabdomere not in cytoplasm.
We have corrected this title of graph in the new version (Figure 3E).

- Fig3CDE: results redundant with Fig1C
Figure 1C shows many Rh1 positive vesicles (RPVs) were detected in the photoreceptor cell bodies of newly eclosed adult mutants, which explains the sensitivity reduction phenotype in adult flies. On the contrary, Figure 3CDE show the time course of Rh1 trafficking in late pupa, before eclosion. These panels not only confirm the importance of deglycosylation for rapid Rh1 transport, together with the time courses of Rh1 deglycosylation (Figure 3AB), they also provide several additional suggestions. First, the initiation of Rh1 expression is normal in *dmppe* mutant, as the expression and distribution of Rh1 is similar at 68% PD in both wild type and mutant. Second, Rh1 undergoes deglycosylation during its trafficking to rhabdomere. Third, the slow transport of glycosylated Rh1 in the mutant is not due to abnormal rhabdomere differentiation or morphogenesis, as the

rhabdomeral morphology was normal in Figure 3CDE. Due to these reasons, we still keep this data in the new version (New Figure 3CDE).

- p9: "In two-day-old dark-reared flies, dmppe mutants displayed normal ERG responses (Fig S1 A), with the sensitivity to light at the wild-type level (Fig S1 B)." Quantification of the plateau and the prolonged depolarization afterpotential (PDA) is necessary to conclude that rh1 has a normal behavior in ERG. Quantification of ON and OFF transients would complete the ERG analysis
We have performed additional quantifications and have included the results in supplementary figure 1. The mutant did have normal ERG in term of amplitude (new Supplementary Figure 1B), ON and OFF transients (new Supplementary Figure 1C), light sensitivity (new Supplementary Figure 1D), speed of termination (Supplementary Figure 1E), and the minimal light intensity for PDA (Supplementary Figure 1F). Thus, the deglycosylation of Rh1 is not required for the normal visual signaling.

- Fig4: It is not demonstrated that the decrease light sensitivity of young and old mutant flies, the decrease expression of Rh1 in old mutant flies and the altered rhabdomere structures of mutant flies are due to the defect of rh1 maturation. One could imagine that dMPPE has other target than α-Man-II; and the maturation of Rh1. The decrease of INAD in 20-day-old flies is not commented.
In the new version, we have provided the evidence that 16-day-old, dark-reared mutants have normal Rh1 levels, and normal rhabdomere structure and light sensitivity (Supplementary figure 2). This result indicates that loss of Rh1 is tightly correlated with the other phenotypes including the sensitivity reduction and the rhabdomeral structural defects. It could be that loss of Rh1 leads to the other defects. In old *ninaE* mutants, retinal degeneration occurs independent of light due to the loss of Rh1 (Leonard et al, 1992). The decrease of INAD in 20-day-old flies should be the consequence of retinal degeneration, which has been clarified in the text.

Although we cannot completely exclude another factor that also contributes to the Rh1 loss, so far we did not identify any candidate MPPE target other than α-Man-II which is implicated in the stability of membrane proteins. Thus, the retained oligosaccharide chain is the only likely and the most straightforward explanation for the Rh1 loss.

- Many panels of Fig1 and Fig4 do not correspond to the title of the figures.
We have changed the titles to cover all the panels in the mentioned figures.

-p11: Informations about lethality and nullity of the mutation should be added
In the new version, we have included information about lethality and nullity of the mutations.

-Fig5C, 5D : immunoblotting of dMPPE is necessary as a control of the ectopic expression of dMPPE.
We have added dMPPE western blots in the new version (Figure 5D and E).

- Staining in Fig6A can be improved (especially rh1), and a DAPI staining would be appreciated
In the old Figure 6A, the purpose of Rh1 staining is to show the area of retina. During the revision, we spent much effort to improve the quantity of Rh1 staining. Unfortunately, the Rh1 antibody had problem soaking into the retina of whole-head tissue, although it worked well with isolated ommatidium and sections. Therefore, in the new version, we instead used the antibody 24B10 to show the retina area, and DAPI for nuclei (Figure 6A).

- Fig6D: a costaining to orient the structure is required. Are the rh1 and MPPE staining localized in the forming rhabdomers?
In the new version, rhabdomere bundles are shown by phalloidin staining that reveals F-actin structures. In the new Figure 6E, Rh1 and dMPPE stainings localize in the cytoplasm, but not in the forming rhabdomeres.

- Fig S3 : western blots are not convincing
We repeated these experiments and re-probed the striped membranes for loading control purposes. The new western blots now clearly show that the amounts of several phosphorylated proteins are increased in the *dmppe* mutant (new supplementary figure 4CD).

- Fig7 : numerous results correlate with α-Man-II; as the mediator of dMPPE for rh1 deglycosylation. Rescuing rh1 deglycosylation in dMPPE mutant with a constitutively active form of α-Man-II; (such as a non phosphorylable form) would prove that α-Man-II; is the mediator of dMPPE for rh1 deglycosylation.
To demonstrate that α-Man-Ⅱ is the mediator of the dMPPE function in Rh1 deglycosylation, we have first identified a phosphorylation site of α-Man-Ⅱ. We introduced a myc-tagged α-Man-Ⅱ into both wild-type and *dmmpe* mutant flies (new Figure 8B). After immuno-purifying the protein from both flies, we digested the samples with trypsin. Through mass spectrum analyses, we identified serine-73 as the sole phosphorylation site of α-Man-Ⅱ. At the end, we introduced a mutant variant of α-Man-Ⅱ that has serine-73 mutated to glycine (S73G) into the *dmmpe* mutant background. This α-Man-Ⅱ^{S73G} protein successfully rescued the Rh1 deglycosylation defects (new Figure 9E), confirming that dMPPE mediated Rh1 deglycosylation by dephosphorylating α-Man-Ⅱ (new Figure 9F).

- p16: generating the double mutant α-Man-II / edem1, edem2, png1, α-Man-Ⅰ, α-Man-Ⅱb and CG14015 are more prone to identify the glycosidase that further remove the remaining GnMan3Gn2 yielded by α-Man-II; hydrolysis.
In the previous version, we obtained/generated mutant flies of known and predicted glycosidase gene to screen for the glycosidase that removes the remaining GnMan3Gn2 from Rh1. Unfortunately, none of them show a defect in Rh1 deglycosylation (new supplementary figure 7A). During the revision, we did another screen using RNAi lines, and found increased MWs of Rh1 in two independent lines (v42652 and v108043) that aim to knock down the *α-Man-Ⅱb* gene (new Supplementary Figure 7B). Interestingly, those Rh1 MWs were smaller than those in *dmppe* and *α-Man-Ⅱ* mutants. These results may suggest that *α-Man-Ⅱb* removes the remaining GnMan3Gn2 yielded by the α-Man-Ⅱ hydrolysis.

Previously we did not detect an Rh1 defect in an *α-Man-IIb^{NP5401}* mutant that has a P element inserted in the 5'-UTR of the *α-Man-Ⅱb* gene. Since the insertion site is 15 Kb from the first exon, the insertion may not really disrupt the gene. The lethality observed in this mutant could be due to a background mutation or the disruption of a neighboring gene. We will further study the role of *α-Man-Ⅱb* in the future.

-p19: "it renders Rh1 more sensitive to endocytic degradation, and could cause morphological and functional defects in the photoreceptor cells of old dmppe flies." This hypothesis does not correspond to the conclusion of a recent paper Chinchore et al 2009 which describes that accumulation of rhodopsine1 in endocytic compartment, due to an absence of degradation for exemple, elicit cell death of the photoreceptor
Our hypothesis is that loss of Rh1 leads to slow retinal degeneration. In the mutant, glycosylated Rh1 is rapidly degraded upon endocytosis (Figure 4E and F). The consequence of Rh1 degradation is a slow loss of Rh1, which at the end leads to retinal degeneration. Retinal degeneration also occurs in *ninaE* mutants that have reduced Rh1 levels (Leonard et al, 1992).

Accumulated of Rh1 in endocytic pathway, either by increasing Rh1 endocytosis rate or by blocking rh1 degradation, also triggers photoreceptor death (Chinchore et al, 2009). However, it occurs through a different pathway of cell death. Our hypothesis on the Rh1 loss-triggered photoreceptor degeneration does not conflict with the conclusion of Chinchore's paper.

Referee #2 (Remarks to the Author):

I highly recommend this paper published in EMBO Journal, but they should address some specific points.

1) Regarding Fig 1C, I wonder how quickly authors fixed flies after they moved dark reared flies to light condition (I guess they fixed flies with light). As within 20mins after illumination, many RPPs are formed, 10-20mins difference for fixation speed gives a big difference in RPPs number. In other words, it there really many RPPs in complete dark reared dMPPE mutant?

Satoh et al. showed that RPPs will form in wild type flies after 10-20 mins strong illumination(Satoh & Ready, 2005). To avoid light-induced Rh1 endocytosis, flies were reared in dark from early pupal stage. Before fixation, flies were collected and dissected in dim red light, and the heads were fixed in the dark. The total time of exposure to dim red light was less than 5 minutes. This information is described in Materials and Method.

3) There is no information about anti Man II antibody.
We apologize for the confusion. We actually did not generate an anti-α-Man-Ⅱ antibody. Commercial anti-human α-Man-Ⅱ antibodies do not recognize the *Drosophila* α-Man-Ⅱ. In our experiments we used a Myc antibody to stain the myc-tagged α-Man-Ⅱ (old Figure 7B and C, new Supplementary Figure 6). Although we mentioned this information in the figure legend (old Figure 7B), it could still cause confusion in the text and figure. Thus, we have rewritten the text and re-labeled the figures to avoid the confusion.

4) Regarding dMPPE localization. They showed partial colocalization of dMPPE with calnexin and Syntaxin6 in dMPPE tranfected HEK293 and stated dMPPE predominantly exists in ER and partial resides in Golgi. However, Fig 6B and D show endogenous dMPPE localizes on Golgi bodies in fly photoreceptor. I guess over expression study often overflows proteins from its real destination. I think they can say more strongly dMPPE localization on Golgi in fly photoreceptors.
The reviewer is right about the overflow of over-expressed proteins. To reveal the localization of endogenous dMPPE, we co-stained dissected ommatidium with both anti dMPPE antibody and ER/Golgi markers (New Figure 6CD). The results showed that endogenous dMPPE predominately resides in Golgi bodies.

5) Why does dMPPE mutant photoreceptor degenerate? Is this because of quick Rh1 degradation in dMPPE mutant? Is this light dependent degeneration ?
In the mutant, the endocytic glycosylated Rh1 is easy to degrade (New Figure 4E and F) and the consequence of Rh1 degradation is a slow loss of Rh1 (New Figure 3G and Figure 4B), which at the end leads to retinal degeneration. After the Rh1 endocytosis was blocked by light deprivation, no retinal degeneration was observed in the *dmppe* mutant (Supplementary figure 2).

6) Typo P8 middle (Fig3C ->3B)s
We have corrected this mistake.

Referee #3 (Remarks to the Author):

Major Comments:

1. Figure 7, panels F-H. These data are the key findings related to processing of Rh1. First of all, panels G and H are mislabeled in the legend or figure. But, regardless, the following is clear, and very interesting: Rh1 fails to be processed in the Man-II mutant just as it remains unprocessed in the mppe mutant. This result indicates that Man-II is necessary for deglycosylation of Rh1. However, it does not necessarily mean that Mppe is necessary for Man-II activation. In fact, the panel labeled "G" in Figure 7 suggests otherwise. The last two lanes of this panel show that Man-II reduces the molecular weight of Rh1 to the same extent, with or without MPPE. It is very difficult, however, to figure out what was actually done in panel G. The methods section does not describe the experiment and the figure legend is extremely minimal. I'm assuming that Rh1 from mppe mutant was treated with some form of recombinant MPPE and Man-II in vitro, not in vivo. If Man-II requires MPPE activation, why is it active on Rh1 in vitro? The result in Panel H is also confusing. Man-II should reduce the glycan to a smaller core than the 2,3-Mannosidase, yet incubation with the 2,3-Mannosidase almost reduces the mass all the way to the deglycosylated form. Can the authors propose a glycan structure or structures that might satisfy the data? Also, essentially no details are given for how the exoglycosidase treatments were done.
Firstly, we apologize for the mislabeling. We corrected the labeling and rewrote the text and figure legend to avoid confusion.

In the old Figure 7F, we showed that the MW of Rh1 from *α-Man-Ⅱ* mutant was as similar as that of *dmppe* mutant (New Figure 9C) and the MW of Rh1 was reduced after digestion with PNGase

(New Figure 9D). This observation demonstrates that α-Man-Ⅱ directly mediates the process of Rh1 deglycosylation *in vivo*.

In the old Figure 7G, we showed that the purified recombinant α-Man-Ⅱ could remove the oligosaccharide chains from glycosylated Rh1 *in vitro* (New Figure 9B). Since α-Man-Ⅱ specifically catalyzes the removal of both α-1,3-linked and α-1,6-linked mannoses from GnMan5Gn2 to yield GnMan3Gn2, this process might be disrupted in *dmmpe* mutants.

To elucidate the structure of the remaining oligosaccharide chains in Rh1 from *dmppe* mutants, we performed glycosidase digestion analysis. In old Figure 7H, the MW of Rh1 was reduced after the digestion with α 1-6 mannosidase and further reduced after the incubation with α 1-2,3 mannosidase (new Figure 9A). In old Figure 7I, the structure of GnMan5Gn2 was shown (New Figure 9F). Only one mannose residue (M5 in Figure 9F) can be removed by α 1-6 mannosidase, which specifically removes unbranched α-1,6-linked mannose. However, one N-acetyl-glucosamine residue (G3 in Figure 9F) and two mannose residues (M2 and M4 in Figure 9F) can be removed by α 1-2,3 mannosidase. This result consists with the structure of GnMan5Gn.

To prove that the Rh1 deglycosylation defect in *dmppe* mutants is really due to the phosphorylation of α-Man-Ⅱ, we introduced a mutant variant of α-Man-Ⅱ into transgenic flies, which has the phosphorylation site serine-73 mutated to glycine (S73G). This mutant α-Man-Ⅱ, but not the wild-type protein, successfully rescued the defect of Rh1 deglycosylation in the *dmppe* mutant background (New Figure 9E). This result confirms that dMPPE promotes Rh1 deglycosylation by mediating the dephosphorylation of α-Man-Ⅱ (New Figure 9F).

In the old Figure 7G, the last two lanes of this panel show that recombinant α-Man-II reduces the molecular weight of Rh1 to the same extent *in vitro*, with or without recombinant dMPPE. The explanation is that the purified recombinant α-Man-II protein is not phosphorylated.

In the new version, we reorganized the figure and rewrote the text and figure legends to make the manuscript easy to read.

2. A major piece of the mechanism is missing. How is the glycan removed following Man-II action? The authors state that Drosophila PNGase mutants (png) are not deficient in deglycosylation, so that would tend to rule out an ERAD-type mechanism. Without proposing or identifying a mechanism for deglycosylation, it remains unclear how Man-II regulation is relevant for the deglycosylation process and the data becomes more of a preliminary report than a definitive finding.

To identify the glycosidase that removes the remaining GnMan3Gn2 yielded by α-Man-Ⅱ hydrolysis, we have conducted another screen using RNAi lines that knock down known and predicted glycosidase genes. We found that in two independent RNAi flies targeting *α-Man-Ⅱb* the Rh1 MW is larger than the wild type, but smaller than that in the *α-Man-Ⅱ* mutant (New Supplementary Figure 7B). It is likely that *α-Man-Ⅱb* removes the remaining GnMan3Gn2 from Rh1. The *α-Man-IIb*^{NP5401} mutant that we previously tested may not be real null of *α-Man-Ⅱb* (see responses to the reviewer #1).

3. The proteomic data presentation is insufficient. Is it true that peptides belonging to no other proteins were found in the gel slices at 130 and 51 kD? What is the percent coverage for Man-II? What is the confidence level for the protein id? As presented, it seems that Man-II may have been pursued because of it's role in glycan processing rather than because of the strength of the proteomic id. The quality of the anti-phosphopeptide antibody blots in supplement Fig. S3 does not clearly support that bands at 130 and 51kD are of interest. So, one has to assume that the selection of these targets was based on the silver stain (is it a silver stain?) in panel A of figure 7. The id of these proteins as phosphoproteins is based solely on metal ion affinity chromatography, which can also enrich for sulfated, sialylated, or mannose-phosphorylated proteins. Therefore, without actually mapping a phosphorylation site on the targets, it is not clear how to test whether phosphorylation detected in vitro is functionally relevant in vivo.

In proteomic data, the percent coverage for α-Man-II is 20%, the score is 74, and the expect value is 0.0093. In the new version, we have clearly described the information in Supplementary Table 1.

According to the reviewer's suggestion, we have mapped a single phosphorylation site of α-Man-Ⅱ to the residue serine-73 using MS. At the end, we introduced a mutant variant of α-Man-Ⅱ that has serine-73 mutated to glycine (S73G) into the *dmmpe* mutant background. This α-Man-Ⅱ^{S73G} protein successfully rescued the Rh1 deglycosylation defects (new Figure 9E), confirming that dephosphorylation of the mapped site is critical for the α-Man-Ⅱ activity (new Figure 9F).

Minor Comments:

1. Fig. 1, Panel B: It is not clear from the methods section what is meant by "Rela light sensitivity (%)." Is this based on response at a single light intensity or averaged over many intensities? If so, what intensity or intensities were chosen? Panel C: Do the authors mean "RPV" or "RPP" for the y-axis? Panel D: The authors should provide more context for the EM. The significance of the inset is not clear.
In previous version, in Figure 1B, "Rela light sensitivity (%)" is relative light sensitivity. The relative light sensitivity of a photoreceptor is defined as I_{WT}/I, where I_{WT} represents the mean light intensity of triggering a detectable response in the wild-type eye, and I is the lowest light intensity for the individual eye examined. For each genotype and condition, 12 flies were examined and the relative sensitivities were averaged to obtain a mean. The standard error of the mean (SEM) was calculated and presented as error bar in the figures.

In Figure 1C, the y-axis represents the number of rhodopsin positive vesicles per ommitidium. In the new version, to avoid confusion, we have used "RPV" in the text, figures and figure legends.

In the new version, we have described more about the EM, and have explained the implication of information from the inset (New Figure 1D), i.e., Rh1 could be retained in MVBs during secretion.

2. Fig. 3, Panel A,B: Something is mislabeled here. Panel E: Shouldn't the y-axis be "% of signal in Rhabdomere" not "Cytop."
We have corrected these mistakes in the new version.

3. Fig. 5, Panel E: Again, shouldn't "RPP" be "RPV?"
We have changed "RPP" to "RPV" for the consistence.

4. Supplement Fig. 2 is not necessary.
In the new version, we have removed this data.

5. Supplement Fig. 3: What are the arrows meant to indicate?
In the old Supplementary Figure 3, the arrows pointed to the bands whose amount increased in the dmppe mutant (new Supplementary Figure 4). This has been clarified in the new version.

6. Numerous instances of improper words are found throughout the manuscript. A rigorous proof-reading by a native English speaker would help the readability of the manuscript.
We thank the reviewer for this suggestion. With the help of a native English speaker, we have polished the writing and have corrected the spelling and grammatical errors.

Chinchore Y, Mitra A, Dolph PJ (2009) Accumulation of rhodopsin in late endosomes triggers photoreceptor cell degeneration. *PLoS genetics* **5**(2)**:** e1000377

Leonard DS, Bowman VD, Ready DF, Pak WL (1992) Degeneration of photoreceptors in rhodopsin mutants of Drosophila. *Journal of neurobiology* **23**(6)**:** 605-626

Satoh AK, Ready DF (2005) Arrestin1 mediates light-dependent rhodopsin endocytosis and cell survival. *Curr Biol* **15**(19)**:** 1722-1733

3rd Editorial Decision 28 June 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2011-77041R. It has

now been seen again by referees 2 and 4, whose comments are enclosed below. As you will see, both find the manuscript to be substantially improved and now support publication in the EMBO Journal, pending a couple of remaining minor text changes. I would therefore ask you to revise the manuscript according to their comments; I hope then we should be able to accept the study.

I also have one request from the editorial side. We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide a single PDF file comprising the original, uncropped and unprocessed scans of all gels used in the figures? These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. This PDF will be published online with the article as a supplementary "Source Data" file. Please let me know if you have any questions about this policy.

I look forward to receiving the revised version of your manunscript.

REFEREE REPORTS

Referee #2 (Remarks to the Author):

The authors have answered to all the reviewer's comments. I am satisfied with their modified version to the exception of one statement about ninaA mutant in their discussion that should be corrected for clarity.

In ninaA mutant, glycosylated rh1 accumulates in the ER, which in turn activate the UPR. As a contrary to what it is stated in the discussion paragraph, Mendes et al. have shown that photoreceptor remain intact despite accumulation of Rh1. Furthermore photoreceptor submitted to mild ER stres trigger a protective response which protects them form apoptosis. Thus ninaA mutant could be used to study the function effects of Rh1 deglycosylation. Nevertheless in dmppe as contrary to ninaA mutant, Rh1 has no problem exiting the ER which makes it an interesting model. The authors need to correct the statement in the discussion.

"Although in several mutants, such as ninaA, Rh1 remains almost fully glycosylated, it is misfolded and accumulates in the ER (Colley et al, 1991). These flies undergo ER stress-mediated photoreceptor degeneration (Mendes et al, 2009) and thus are not appropriate for the study of functional effects of the Rh1 deglycosylation. For this purpose, the dmppe mutant appears to be a good system of study, since the glycosylated Rh1 has no problem exiting the ER in this mutant."

The paragraph could be corrected as follows:
Although in several mutants, such as ninaA, Rh1 remains almost fully glycosylated, it is misfolded and accumulates in the ER (Colley et al, 1991). Rh1 accumulation in the ER triggers an ER stress, which in turn activates the unfolded protein response (Mendes et al, 2009). In contrast in dmppe mutant, glycosylated Rh1 has no problem exiting the ER, which makes it a good model of study.

Referee #4 (Remarks to the Author):

The authors have appropriately addressed most of the comments raised in the previous review of this manuscript. However, I am troubled by a sentence in their responses to Referee #3. In response to Comment #2, the authors state "It is likely that α-Man-IIb removes the remaining GnMan3Gn2 from Rh1." I am unaware of any data that would support such an activity for this enzyme. Fortunately, neither this sentence, nor any other sentence with a similar meaning can be found in the manuscript.

Looking carefully now at the data in this manuscript and at the data from earlier publications cited by the authors, it is not clear to me that the entire glycan is actually removed from Rh1. The data in this manuscript and all of the published data cited by the authors is entirely consistent with the retention of a Man2Gn2 or even a Man1Gn2 core that may be insufficient to cause a mass shift detectable by SDS-PAGE in comparison to PNGaseF treated Rh1. While the point of this manuscript is not to define the glycan structure on Rh1, it is confusing to refer to Rh1 as

"deglycosylated" when there is no evidence for such a processing event (other than ERAD which is not invoked here). I would urge the authors to refer to the mass-shifted Rh1 as "extensively processed" or "extensively trimmed" until the exact glycosylation status of mature Rh1 has been determined by direct structural analysis. Unless the authors can cite data (which I can't find) that directly demonstrates the lack of a glycan on mature Rh1, it would be good to avoid perpetuating the concept of protein deglycosylation in the secretory pathway.

A minor comment: The biological and commercial source of the mannosidases used to digest Rh1 should be given in the Materials and Methods section.

2nd Revision - authors' response 30 June 2011

Referee #2:

The authors have answered to all the reviewer's comments. I am satisfied with their modified to the exception of one statement about ninaA mutant in their discussion that should be corrected for clarity.

In ninaA mutant, glycosylated rh1 accumulates in the ER, which in turn activate the UPR. As a contrary to what it is stated in the discussion paragraph, Mendes et al. have shown that photoreceptor remain intact despite accumulation of Rh1. Furthermore photoreceptor submitted to mild ER stres trigger a protective response which protects them form apoptosis. Thus ninaA mutant could be used to study the function effects of Rh1 deglycosylation. Nevertheless in dmppe as contrary to ninaA mutant, Rh1 has no problem exiting the ER which makes it an interesting model. The authors need to correct the statement in the discussion.

"Although in several mutants, such as ninaA, Rh1 remains almost fully glycosylated, it is misfolded and accumulates in the ER (Colley et al, 1991). These flies undergo ER stress-mediated photoreceptor degeneration (Mendes et al, 2009) and thus are not appropriate for the study of functional effects of the Rh1 deglycosylation. For this purpose, the dmppe mutant appears to be a good system of study, since the glycosylated Rh1 has no problem exiting the ER in this mutant."

The paragraph could be corrected as follows:
Although in several mutants, such as ninaA, Rh1 remains almost fully glycosylated, it is misfolded and accumulates in the ER (Colley et al, 1991). Rh1 accumulation in the ER triggers an ER stress, which in turn activates the unfolded protein response (Mendes et al, 2009). In contrast in dmppe mutant, glycosylated Rh1 has no problem exiting the ER, which makes it a good model of study.

The reviewer's point is well taken. We have revised the paragraph as suggested.

Referee #4:

The authors have appropriately addressed most of the comments raised in the previous review of this manuscript. However, I am troubled by a sentence in their responses to Referee #3. In response to Comment #2, the authors state "It is likely thatα-Man-IIb removes the remaining GnMan3Gn2 from Rh1." I am unaware of any data that would support such an activity for this enzyme. Fortunately, neither this sentence, nor any other sentence with a similar meaning can be found in the manuscript.

The related data is in the supplementary Figure 7B, which shows incomplete deglycosylation of Rh1 in two RNAi lines (v42652 and v108043) of α-Man-IIb. However, we agree with the review on that the data is not sufficient to prove that α-Man-IIb removes the remaining GnMan3Gn2. The data only shows that α-Man-IIb has a role in the further trimming of the oligosaccharide chain left by α-Man-II. Fortunately, as the reviewer pointed out, the overstatement is not found in the manuscript.

Looking carefully now at the data in this manuscript and at the data from earlier publications cited

by the authors, it is not clear to me that the entire glycan is actually removed from Rh1. The data in this manuscript and all of the published data cited by the authors is entirely consistent with the retention of a Man2Gn2 or even a Man1Gn2 core that may be insufficient to cause a mass shift detectable by SDS-PAGE in comparison to PNGaseF treated Rh1. While the point of this manuscript is not to define the glycan structure on Rh1, it is confusing to refer to Rh1 as "deglycosylated" when there is no evidence for such a processing event (other than ERAD which is not invoked here). I would urge the authors to refer to the mass-shifted Rh1 as "extensively processed" or "extensively trimmed" until the exact glycosylation status of mature Rh1 has been determined by direct structural analysis. Unless the authors can cite data (which I can't find) that directly demonstrates the lack of a glycan on mature Rh1, it would be good to avoid perpetuating the concept of protein deglycosylation in the secretory pathway.

We referred to the mature Rh1 as "deglycosylated" because this was also supported by previously SDS-PAGE analyses and was mentioned in several literatures (Huber et al, 1990; Katanosaka et al, 1998). However, we agree with the reviewer that SDS-PAGE may not be able to tell the existence of a short oligosaccharide chain. Since direct evidence (such as peptide mass spectrum) for the complete deglycosylation of Rh1 is lacking, we are now referring to the mature Rh1 as "extensively trimmed" or "extensively processed" in the revised version, as suggested by the reviewer.

A minor comment: The biological and commercial source of the mannosidases used to digest Rh1 should be given in the Materials and Methods section.

We have added this information into the Materials and Methods section.

We would like to thank the reviewers for their additional comments, and hope you will now find the manuscript acceptable for publication in *EMBO J*.

Huber A, Smith DP, Zuker CS, Paulsen R (1990) Opsin of Calliphora peripheral photoreceptors R1-6. Homology with Drosophila Rh1 and posttranslational processing. *The Journal of biological chemistry* **265**(29)**:** 17906-17910

Katanosaka K, Tokunaga F, Kawamura S, Ozaki K (1998) N-linked glycosylation of Drosophila rhodopsin occurs exclusively in the amino-terminal domain and functions in rhodopsin maturation. *FEBS letters* **424**(3)**:** 149-154

