JCB

PERK prevents rhodopsin degradation during retinitis pigmentosa by inhibiting IRE1-induced autophagy

Ning Zhao, Ning Li, and Tao Wang

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October 13, 2022

Re: JCB manuscript #202208147

Dr. Tao Wang
National Institute of Biological Sciences
No. 7, Park Road, Zhongguancun Life Science Park, Changping District,
Beijing 102206
China

Dear Dr. Wang,

Thank you for submitting your manuscript entitled "PERK prevents degradation of rhodopsin and retinal degeneration by inhibiting IRE1/XBP1-induced ER-phagy". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that reviewers appreciated the conceptual advance linking the effect of Rh1 misfolding with diminishes wt Rh1. However, multiple reviewers felt that the observations on autophagy required greater evidence for specific targeting to the ER, in order to support the conclusions on ER-phagy. A revised manuscript should include data to address these points by Reviewers 1 and 3. In addition points by Reviewer 1 regarding overexpression of wt Rh1, Xbp1 and USP15 will make important improvements to this work. Last, points 2-5 by Reviewer 3 should be addressed. While all comments should be addressed in some form, additional data beyond those mentioned here are not required in a revision.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

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If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Hong Zhang
Monitoring Editor
Journal of Cell Biology

Tim Fessenden
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Rhodopsin (rho) mutations that activate the Unfolded Protein Response (UPR) underlie autosomal dominant Retinitis Pigmentosa (adRP), a dominant genetic disease with age-related retinal degeneration. How the UPR pathways affect the wild type versus the mutant Rho proteins remains poorly understood. Here, Zhao and Wang employ Drosophila to investigate the effects of UPR signaling on these Rho alleles. They find that Perk and eIF2Ba loss reduces the wild type Rho protein in the Rho P37H/+ model of adRP by hyper-activating the ire1-xbp1-ER phagy axis, ultimately causing accelerated retinal degeneration. On the other hand, these photoreceptors regulate the mutant Rho P37H protein through a distinct mechanism that involves proteasomal degradation. The authors further validate the results with an independent adRP model, ninaE G69D.

Overall, this is an impressive study that combines a multitude of approaches, ranging from an unbiased EMS mutagenesis screen, the employment of fluorescent reporters, gene expression profiling, proteomics, and more. The experiments are well controlled, and the derived conclusions are appropriate. The results highlight the importance of Perk in suppressing the activation of the ire1-xbp1-ER phagy signaling axis, which is essential to maintain the levels of the wild type Rho protein and suppress retinal degeneration. Perhaps because the study is quite expansive, the authors gloss over some details that could draw further interest from the research community. In addition, there are a couple of citations missing. Below are specific comments for the authors' consideration along these lines:

1. The authors show that Rho P37H flies reduce wild type Rho protein levels, and such reduction correlates with accelerated retinal degeneration. Do the authors think the reduction of wild type Rho is a "cause" for retinal degeneration? What would happen to retinal degeneration if the authors overexpress the wild type Rho transgene in the Rho P37H background?
2. On page 3, the authors introduce that PERK inhibits eIF2a, and then activates ATF4. It is worth noting that ATF4 is not the only transcription factor induced by PERK. The authors might want to mention that Xrp1 is another transcription factor induced downstream of PERK in Drosophila (Brown et al., 2021 eLife PMID 34605405).
3. The identification of an eIF2Ba allele as something that enhances wild type Rho degradation in Rho P37H is interesting. However, the authors don't introduce what is known about eIF2B in the Perk-eIF2a signaling pathway. Therefore, some introduction would be helpful to the readers.
4. Is the newly identified eIF2Ba allele a simple loss of function allele? Since eIF2B should be required for most (if not all) protein synthesis, simple loss-of-function clones may not be able to survive. The authors may want to comment on this.
5. One possible way the eIF2Ba allele behaves like Perk loss of function would be if the mutant eIF2Ba is immune to inhibition by phosphor-eIF2a. There is already a lot of literature on eIF2B as a phosphor-eIF2a target. More recently, there have been lots of studies on a compound called ISRIB which makes eIF2B indifferent to phosphor-eIF2a. Perhaps the authors could use the existing structure models to predict what the mutation in eIF2Ba is affecting. It would draw a lot of interest from researchers interested in Perk-eIF2a signaling.

6. The authors show that retinal degeneration of Rh1 P37H-GFP flies is aggravated in the Perk mutant background. A similar conclusion had been reported with ninaE G69D flies before (Vasudevan et al., 2020 PMID 32938929; see Figure 5 in that paper). The authors should acknowledge this.
7. Looking at Ref(2)P/P62 levels (Figure 5), the authors show effects reversed by ire1 RNAi. Does Xbp1 RNAi also reverse the effects?
8. The authors show the involvement of general autophagy regulators such as Ref(2)P, atg1, atf9 and atg18. Yet, they see that Rh1 P37H-GFP Perk RNAi promotes ER phagy, and not general autophagy of other subcellular organelles. Do the authors have an explanation for this specificity? Are there specific ER phagy genes induced downstream of ire1-xbp1?
9. The authors speculate that ER protein ubiquitination drives autophagy. Are there any ER-associated ubiquitin ligases that are induced by ire1-xbp1? How would ubiquitination of ER proteins increase in response to ire1-xbp1 signaling?
10. Also, regarding the specific induction of ER-phagy, is USP15-31 associated with the ER?
11. The authors should provide an excel file of gene expression profiling results as a supplementary file.
12. Depositing the sequence files to NIH GEO is strongly recommended.

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, the authors established a fluorescence reporter system to monitor mutant and wild-type rhodopsin in Drosophila eye. They found that Perk signaling plays a critical role in maintaining rhodopsin homeostasis by inhibiting Ire1-induced ER-phagy. Their findings suggest that modulation of Perk activities may provide a potential therapeutic intervention for ER stress-related neuropathies. The authors have generated an impressive amount of data to understand the interaction between Perk signaling and rhodopsin homeostasis. However, the underlying mechanism of how Perk antagonizes Ire1-induced ER-phagy remains elusive.

Major
1.The authors claim that Rh1P37H-GFP accumulated exclusively in the ER whereas wild-type Rh1-RFP localized to the rhabdomeres. However, Rh1ph expression pattern are quite different in Fig 1D, especially the one that co-stained with INAD.

2. Similarly, the pattern of Rh1ph-GFP are quite different in Fig S1a and S1b, suggesting that Xbp1-mCherry may affect the distribution of Rh1ph mutant. Importantly, instead of the fluorescent intensity of Xbp1 and ATF4, their activation should be determined by examining the localization of Xbp1 and ATF4 in nucleus.

3. The authors should explain why a lower MW Rh1ph (not as claimed in line159) was observed in perk and eIF2Bα mutant flies (Fig 2c, Fig 3).

4. While their data suggest that ref(2)p acts as an autophagic receptor in Ire1-mediated Rh1 degradation, a direct interaction between ref(2)p and Rh1 should be included.

5. Results line345 is not consistent with the data presented in Fig7g-h. USP15-3 overexpression does not seem to affect levels of wild-type Rh1 in Rh1P37H-GFP perkRNAi flies. How about the levels of Rh1ph mutant?
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Minor
MW should be included in all western blots.

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript, the authors establish many useful new tools to interrogate the role of various ER stress signaling branches in Drosophila models of human eye diseases including autosomal dominant retinitis pigmentosa. In a mutant rhodopsin-induced degradation of wild-type rhodopsin eye model, a large-scale chemical genetic screen shows that PERK-eIF2a signaling is necessary to maintain rhodopsin homeostasis. Loss of PERK prevents proteasomal degradation of mutant rhodopsin, which leads to autophagic degradation of wild type rhodopsin. I think the story is generally interesting and important, but some of the claims may be overinterpretations, some of the data are inconsistent and I would like to see some further developments (please see below) before recommending this work for publication. Also, English language usage must be improved.

1. As for PERK loss-induced changes on a Rh1 P37H background: p62 is upregulated and colocalizes with both wt mutant Rh1, while autophagy is induced and Atg8a colocalizes with wt but not mutant Rh1. P62 is a selective receptor that aggregates ubiquitinated cargo, so I miss testing where the ubiquitinated cargo is found that "recruits" p62: on the surface of specific subdomains of the ER? Everywhere on the surface of ER? Please also discuss how it may get there - e.g., ubiquitinated proteins get stuck while being transported out of the ER?

2. Most importantly, not only the level of ER-GFP (Fig S6b) but also mito-GFP (S6c) decreases in a statistically significant manner, and there is a tendency of decreasing cytosolic GFP as well (S6e - I wonder if this obvious change would also become statistically significant if more western blots are done and evaluated, as currently sample number n is only 3). How do we know that wild-type Rh1 is (only) in the ER when it is captured by autophagy? Direct evidence for its selective ER-phagy is lacking. While I'm fine with the claim that PERK loss induces (general?) autophagy in Rh1 mutant expressing cells that leads to a decline in wild-type Rh1 level, I suggest toning down a bit the involvement of a specific ER-phagy pathway (including the abstract, say, "induction of autophagy including ER-phagy") unless more convincing further data can be added to support its ER selectivity and exclusivity.

3. Transcriptional upregulation of a few selected Atg genes is shown in fig 5f, but some of the most important ones are missing: Atg9 and Atg8a are rate-limiting for autophagosome initiation/numbers and size, respectively, and a number of papers showed that Atg8 family genes are usually the most highly upregulated ones not only in yeast but also in Drosophila. Please also test these two genes/mRNA levels in your setting.

4. Fig S6a: arrows point to "empty-looking" vesicles, which are unlikely to be autophagosomes. Autophagosomes have two limiting membranes and contain cytoplasm, including organelles such as ER fragments. Please provide high-mag images of autophagosomes that fulfil these criteria or refrain from calling these vesicles autophagosomes.

5. It would be interesting to test the effect of p62 loss on the ninaE model phenotypes - is it similar to its effects in the Rh1 mutant model? Also, why is the loss of p62 rescuing (fig 6e) - is it the loss of ubiquitinated protein aggregation, the loss of their autophagy, or both? Published flies having CRISPR mutant p62 that cannot bind Atg8 to deliver cargo for autophagy could be used to test this (PMID: 35184662), or at least discuss this please.

Minor

6. I don't see p62 puncta in ninaE mutant cells (fig 8c), which is at odds with the statistics (8d). Please clarify.

7. While p62 upregulation in perk RNAi is really convincing in immunostainings (fig 5 d,e), differences seem much smaller on the current wb/fig 5b then the statistics/fig 5c. Please use a more representative blot.

8. The authors summarize their findings at the end of the intro, in 16 lines. I think it's too long for an introduction chapter.

9. There are studies showing that PERK/eIF2a induces autophagy in Drosophila and mammalian cells while autophagy is induced by loss of PERK and accumulation of mutant rhodopsin in the present model, with the IRE1/XBP1 signaling being important (although it's not clear how). Please elaborate a bit on this in the discussion.

10. Based on the Methods section, normality of data distribution was not tested. Please evaluate the normality of all datasets, because different statistical tests must be used for ones that have non-Gaussian distribution (e.g., u test instead of t test in case of pairwise comparisons).

11. English language usage must be improved. I list here a couple of mistakes from the first part as examples:
L51 which induces targets the...
L58 it is not clear to me what „opposite activating states" means
L97 manor
L102 and whether ER-phagy to maintain cellular
L118 to clear proteins on the ER
And so on...

Reviewer #1 (Comments to the Authors (Required)):

Rhodopsin (rho) mutations that activate the Unfolded Protein Response (UPR) underlie autosomal dominant Retinitis Pigmentosa (adRP), a dominant genetic disease with age-related retinal degeneration. How the UPR pathways affect the wild type versus the mutant Rho proteins remains poorly understood. Here, Zhao and Wang employ Drosophila to investigate the effects of UPR signaling on these Rho alleles. They find that Perk and eIF2Ba loss reduces the wild type Rho protein in the Rho P37H/+ model of adRP by hyper-activating the ire1-xbp1-ER phagy axis, ultimately causing accelerated retinal degeneration. On the other hand, these photoreceptors regulate the mutant Rho P37H protein through a distinct mechanism that involves proteasomal degradation. The authors further validate the results with an independent adRP model, ninaE G69D.

Overall, this is an impressive study that combines a multitude of approaches, ranging from an unbiased EMS mutagenesis screen, the employment of fluorescent reporters, gene expression profiling, proteomics, and more. The experiments are well controlled, and the derived conclusions are appropriate. The results highlight the importance of Perk in suppressing the activation of the ire1-xbp1-ER phagy signaling axis, which is essential to maintain the levels of the wild type Rho protein and suppress retinal degeneration. Perhaps because the study is quite expansive, the authors gloss over some details that could draw further interest from the research community. In addition, there are a couple of citations missing. Below are specific comments for the authors' consideration along these lines:

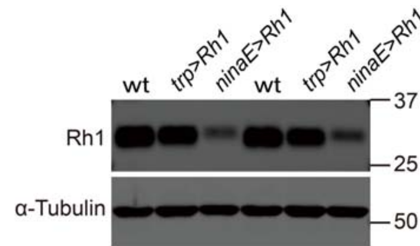
1. The authors show that Rho P37H flies reduce wild type Rho protein levels, and such reduction correlates with accelerated retinal degeneration. Do the authors think the reduction of wild type Rho is a "cause" for retinal degeneration? What would happen to retinal degeneration if the authors overexpress the wild type Rho transgene in the Rho P37H background?

Although we found that flies expressing misfolded Rh1^{P37H}-GFP had less wild-type Rh1, retinal degeneration in these flies was quite slow. By contrast, *Rh1^{P37H}-GFP perk^{12}* mutant flies exhibited rapid photoreceptor cell death with both accumulation of mis-folded Rh1^{P37H} and dramatic reduction of wild-type Rh1. In this case, the accumulation of mis-folded Rh1^{P37H} and/or the reduction of wild-type Rh1 could be the major factor underlying retinal degeneration caused by this type of Rh1 mutation.

Inspired by this comment, we overexpressed wild-type UAS-Rh1 in photoreceptor cells using different Gal4 lines. However, overexpression of wild-type Rh1 via the *GMR* promoter caused a severe rough eye phenotype, as suggested in the literature. Moreover, expression of wild-type Rh1 under the control of *ninaE* or *trp* promoters failed to increase the level of Rh1 protein, which might be because endogenous Rh1 is already saturated in the system. In fact, Rh1 levels even decreased when driven by the *ninaE*

promotor (see western blot), which may be because extra Rh1 is harmful for the development of photoreceptor cells. Nevertheless, we could not overexpress wild-type Rho in the Rho P37H background as suggested.

$Rh1^{P37H}$-GFP $perk^{RNAi}$ flies exhibited dramatic accumulation of mis-folded $Rh1^{P37H}$ and loss of wild-type Rh1. Inhibition of IRE1/XBP1 restored levels of wild-type Rh1 protein in *$Rh1^{P37H}$-GFP $perk^{RNAi}$* flies, but levels of mutant $Rh1^{P37H}$ were unaffected. Therefore, using this system, we examined if the retinal degeneration of *$Rh1^{P37H}$-GFP $perk^{RNAi}$* flies is affected by knocking down *xbp1*. Indeed, knocking down *xbp1* suppressed the ERG loss and delayed retinal degeneration in *$Rh1^{P37H}$-GFP $perk^{RNAi}$* flies, suggesting that increasing wild-type Rh1 slows retinal degeneration. These results support the hypothesis that reductions in wild-type Rho contribute to the retinal degeneration associated with Rho^{P23H} mutants. These results have been added to Figure S3C-E and descriptions have been added to the result section (Line 286-295).



2. On page 3, the authors introduce that PERK inhibits eIF2a, and then activates ATF4. It is worth noting that ATF4 is not the only transcription factor induced by PERK. The authors might want to mention that Xrp1 is another transcription factor induced downstream of PERK in Drosophila (Brown et al., 2021 eLife PMID 34605405).

We also noticed that Xrp1 is another transcription factor induced by PERK in *Drosophila* and thank the reviewer for pointing this out. We added Xrp1 to the introduction section as: "In contrast with the global repression of translation, eIF2α phosphorylation also activates the stress-responsive transcription factors, ATF4 and Xrp1, through selectively enhanced translation (Brown et al., 2021; Harding et al., 1999; Harding et al., 2003). Xrp1 is a newly discovered transcription factor induced downstream of PERK in *Drosophila* (Brown et al., 2021). In addition, UPR signaling activates the IRE1 nuclease, which targets and splices mRNA encoding the transcription factor X-box-binding protein 1 (XBP1), thereby activating it." page 5 (Line 58-64).

Reference:
Brown, B., S. Mitra, F.D. Roach, D. Vasudevan, and H.D. Ryoo. 2021. The transcription factor Xrp1 is required for PERK-mediated antioxidant gene induction in *Drosophila*. *Elife*. 10.

3. The identification of an eIF2Ba allele as something that enhances wild type Rho degradation in Rho P37H is interesting. However, the authors don't introduce what is known about eIF2B in the Perk-eIF2a signaling pathway. Therefore, some introduction would be helpful to the readers.

We added text introducing eIF2B in the Perk-eIF2a signaling pathway on page 4-5 (Line 56-58). “Phosphorylated eIF2α binds and inhibits the guanine nucleotide exchange factor, eIF2B, thereby attenuating eIF2-mediated protein synthesis (Adomavicius et al., 2019) (Kenner et al., 2019).”

Reference:

Adomavicius, T., M. Guaita, Y. Zhou, M.D. Jennings, Z. Latif, A.M. Roseman, and G.D. Pavitt. 2019. The structural basis of translational control by eIF2 phosphorylation. *Nat Commun*. 10:2136.

Kenner, L.R., A.A. Anand, H.C. Nguyen, A.G. Myasnikov, C.J. Klose, L.A. McGeever, J.C. Tsai, L.E. Miller-Vedam, P. Walter, and A. Frost. 2019. eIF2B-catalyzed nucleotide exchange and phosphoregulation by the integrated stress response. *Science*. 364:491-495.

4. Is the newly identified eIF2Ba allele a simple loss of function allele? Since eIF2B should be required for most (if not all) protein synthesis, simple loss-of-function clones may not be able to survive. The authors may want to comment on this.

The newly identified *eIF2Bα* allele (*eIF2Bα^{39}*) is a loss of function allele, as the phenotype of *eIF2Bα^{39}* is absolutely recessive. Although both heterozygous and homozygous *eIF2Bα^{39}* flies are viable, flies with homozygous *eIF2Bα^{39}* photoreceptor cells exhibited Rh1^{37H} accumulation and reduced levels of wild-type Rh1. Further, expression of wild-type eIF2Bα fully restored levels of endogenous Rh1 and reduced levels of Rh1^{P37H}-GFP. We added a sentence in the result section: “Flies heterozygous for any of these *perk* or *eIF2Bα* alleles did not exhibit a phenotype, regardless of whether they express Rh1^{P37H}-GFP or not, suggesting these are loss of function mutations.” on page 10 (Line 161-163).

5. One possible way the eIF2Ba allele behaves like Perk loss of function would be if the mutant eIF2Ba is immune to inhibition by phosphor-eIF2a. There is already a lot of literature on eIF2B as a phosphor-eIF2a target. More recently, there have been lots of studies on a compound called ISRIB which makes eIF2B indifferent to phosphor-eIF2a. Perhaps the authors could use the existing structure models to predict what the mutation in eIF2Ba is affecting. It would draw a lot of interest from researchers interested in Perk-eIF2a signaling.

We thank the reviewer for mentioning this point. It would be interesting to examine the molecular basis of the eIF2Bα mutant. However, as the *eIF2Bα^{39}* mutation is absolutely recessive, it might be hard to explain the idea that “the mutant *eIF2Bα* is immune to inhibition by phosphor-eIF2*α*”. Nevertheless, this point is slightly out of scope of our manuscript. Therefore, we did not further discuss this point in the manuscript.

6. The authors show that retinal degeneration of Rh1 P37H-GFP flies is aggravated in the Perk mutant background. A similar conclusion had been reported with ninaE G69D flies before (Vasudevan et al., 2020 PMID 32938929; see Figure 5 in that paper). The authors

should acknowledge this.

We apologize for overlooking that paper. Now we discuss this paper in the discussion section on Page 31 (Line 615-617), as "Consistent with this, loss-of-function *perk* mutations dramatically accelerate retinal degeneration in fly *ninaE*^{G69D} models (Vasudevan et al., 2020)."

Reference:

Vasudevan, D., S.D. Neuman, A. Yang, L. Lough, B. Brown, A. Bashirullah, T. Cardozo, and H.D. Ryoo. 2020. Translational induction of ATF4 during integrated stress response requires noncanonical initiation factors eIF2D and DENR. *Nat Commun*. 11:4677.

7. Looking at Ref(2)P/P62 levels (Figure 5), the authors show effects reversed by ire1 RNAi. Does Xbp1 RNAi also reverse the effects?

Yes, we examined the levels of Ref(2)P/P62 protein in *Rh1*^{P37H}*-GFP perk*^{RNAi} *xbp1*^{RNAi} flies by western blotting and immunofluorescence. As with *ire1*^{RNAi}, upregulation of Ref(2)P/P62 in *Rh1*^{P37H}*-GFP perk*^{RNAi} flies was also reversed by *xbp1*^{RNAi}. We modified Figure 5B, C and D by adding the *xbp1*^{RNAi} results. Please see the new Figure 5, and corresponding results for details (Page 17, Line 308-309).

8. The authors show the involvement of general autophagy regulators such as Ref(2)P, atg1, atf9 and atg18. Yet, they see that Rh1 P37H-GFP Perk RNAi promotes ER phagy, and not general autophagy of other subcellular organelles. Do the authors have an explanation for this specificity?

As we answered Reviewer 3's question, we used the CRISPR-CAS9 system to generate a *ref(2)P/p62* null mutant fly (*ref(2)P*^{m}) by deleting 4 nt of coding sequences, presumably causing a frame-shift (See the method and supplemental Figure S5I). The *ref(2)P*^{m} mutation itself did not affect autophagy in photoreceptor cells. In the *ninaE*^{G69D} model, the autophagy marker Atg8a was massively induced and accumulated in puncta. However, when *ref(2)P/p62* was knocked out in the *ninaE*^{G69D} cells, the formation of Atg8a puncta was largely abolished. Moreover, as we did for the *ninaE*^{G69D} model, we stained for Atg8a in *Rh1*^{P37H}*-GFP perk*^{RNAi} *ref(2)P*^{RNAi} retina and found the number of Atg8a puncta was dramatically decreased compared with *Rh1*^{P37H}*-GFP perk*^{RNAi} flies (Figure S5I-J, line 397-400, 402-407)(Figure 6C, 332-335). These results strongly support that the massive autophagy seen in both *ninaE*^{G69D} and *Rh1*^{P37H}*-GFP* models are Ref(2)P/P62-dependent autophagy. As P62 is an autophagy adaptor for the ubiquitinated protein, and under ER stress condition most accumulated proteins are ER surface protein (see below), this type of selective autophagy might be specific to ER components.

Are there specific ER phagy genes induced downstream of ire1-xbp1?

As suggested by the reviewer, we surveyed possible ER-phagy genes in the literature

and found that two ER-phagy receptors (*trp1/sec62* and *atl/atl3*) are also induced in *Rh1^{P37H}-GFP perk^{RNAi}* flies. This can be reversed by knocking down *ire1*. We also checked the transcription level of two possible mito-phagy receptors (*nipsnap* and *phb2*), which were not regulated by the ire1-xbp1 pathway. We have added these results to Figure S4I and a description has been added to the results section (Line 343-349).

9. The authors speculate that ER protein ubiquitination drives autophagy. Are there any ER-associated ubiquitin ligases that are induced by ire1-xbp1?

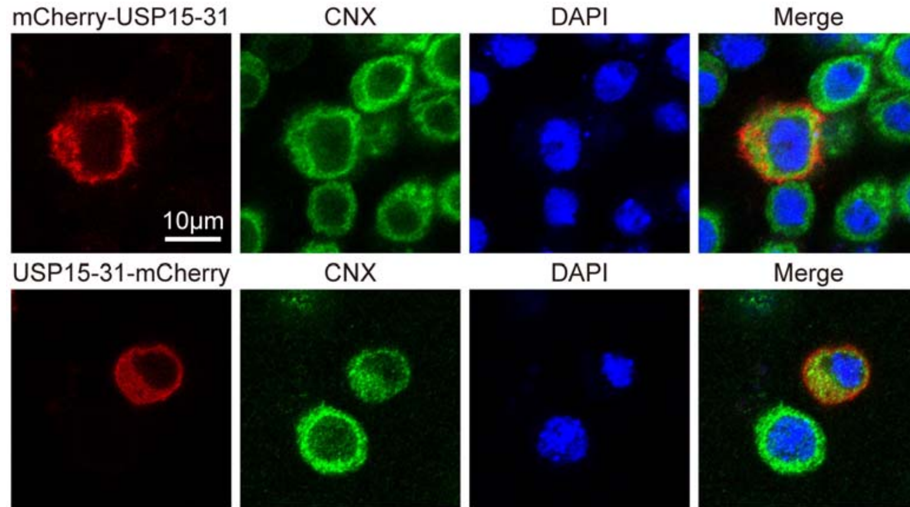
We checked the mRNA levels of *hrd1* and *sordd1*, two transmembrane ubiquitin ligases functioning in the ERAD pathway, and found that transcription of *hrd1* was induced by the IRE1-XBP1 signaling pathway. By contrast, expression of *sordd1* was not affected. These results have been added to Figure 5G and a description has been added to the results section (Line 315-317).

How would ubiquitination of ER proteins increase in response to ire1-xbp1 signaling?

To answer this question, we extracted the membrane component and found that ubiquitination of ER proteins increased in *Rh1^{P37H}-GFP perk^{RNAi}* flies. This increase was reversed by *ire1^{RNAi}* (Figure S4J, line 378-380)

10. Also, regarding the specific induction of ER-phagy, is USP15-31 associated with the ER?

The USP15-31 protein lacks the transmembrane domain and is predicted to localize to the cytosol. To visualize the localization of USP15-3 in S2 cells, we transfecting an N-terminal mCherry tagged USP15-31 and a C-terminal mCherry tagged USP15-31. We confirmed that USP15-31 is a cytosolic protein (see figure below). As we overexpressed USP15-31 for de-ubiquitination, the protein does not necessarily have to be an ER-resident protein, and USP15-31 may not be the *bona fide* factor in the ER stress pathway. It may de-ubiquitinate the accumulated ubiquitinated ER proteins when overexpressed upon prolonged ER stress. We modified the results (line 380-383) for clarification.



11. The authors should provide an excel file of gene expression profiling results as a supplementary file.

We have added three excel files of gene expression profiling results as supplementary files.

12. Depositing the sequence files to NIH GEO is strongly recommended.

We will deposit the sequence files to NIH GEO promptly.

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, the authors established a fluorescence reporter system to monitor mutant and wild-type rhodopsin in Drosophila eye. They found that Perk signaling plays a critical role in maintaining rhodopsin homeostasis by inhibiting Ire1-induced ER-phagy. Their findings suggest that modulation of Perk activities may provide a potential therapeutic intervention for ER stress-related neuropathies. The authors have generated an impressive amount of data to understand the interaction between Perk signaling and rhodopsin homeostasis. However, the underlying mechanism of how Perk antagonizes Ire1-induced ER-phagy remains elusive.

Major

1.The authors claim that Rh1P37H-GFP accumulated exclusively in the ER whereas wild-type Rh1-RFP localized to the rhabdomeres. However, Rh1ph expression pattern are quite different in Fig 1D, especially the one that co-stained with INAD.

This is because the pictures are taken at different focal planes. To avoid confusion, we repeated the assay and now present a new image for the one co-stained with INAD.

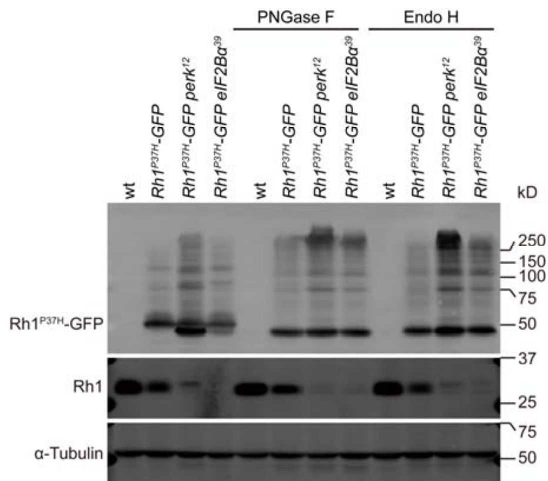
Please see the updated Figure 1D.

2. Similarly, the pattern of Rh1ph-GFP are quite different in Fig S1a and S1b, suggesting that Xbp1-mCherry may affect the distribution of Rh1ph mutant. Importantly, instead of the fluorescent intensity of Xbp1 and ATF4, their activation should be determined by examining the localization of Xbp1 and ATF4 in nucleus.

We repeated this assay by staining for XBP1-mCherry and found that it did not affect the distribution of Rh1^{P37H}-GFP. As suggested by the reviewer, we quantified the nucleus localization of XBP1-mCherry, and concluded that most XBP1-mCherry co-localized with DAPI in the nucleus upon induction by Rh1^{P37H}-GFP. For ATF4, since our ATF4-mCherry reporter only contains the 5' UTR of ATF4 and mCherry, it did not reflect the subcellular localization of ATF4. We added these results to Figures 1G and 1H and line 142-145.

3. The authors should explain why a lower MW Rh1ph (not as claimed in line159) was observed in perk and eIF2Bα mutant flies (Fig 2c, Fig 3).

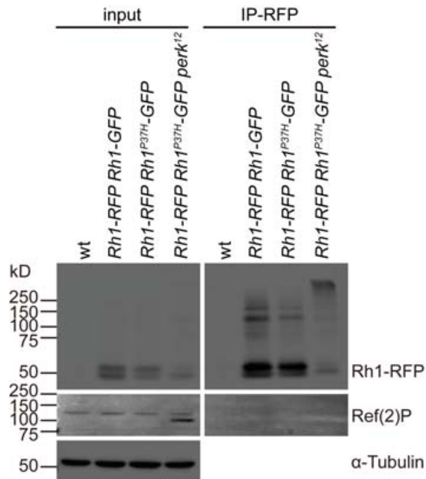
The lower MW Rh1^{P37H}-GFP in *perk*^{12} and *eIF2Bα*^{39} mutant flies is a glycosylation-free band, as digestion with two glycosidases, endoglycosidase H (Endo H) and peptide N-glycosidase F (PNGase F), eliminated the upper band as seen in *perk*^{12} or *eIF2Bα*^{39} animals (See Reviewer Figure below). This might be because the original glycosylation in ER is deficient in *perk*^{12} and *eIF2Bα*^{39} mutant flies due to prolonged ER stress. Although we did not put the figure to main manuscript, we added this description to the results section (Line 166-168).



4. While their data suggest that ref(2)p acts as an autophagic receptor in Ire1-mediated Rh1 degradation, a direct interaction between ref(2)p and Rh1 should be included.

As suggested by the reviewer, we examined the interaction between Rh1 and Ref(2)P/P62 using co-immunoprecipitation in the background of *Rh1*^{P37H}*-GFP perk*^{12}. However, we failed to detect a strong interaction between Rh1 and Ref(2)P/P62. Since

the Ref(2)P/P62 protein is an adaptor that binds both ubiquitinated proteins, including ubiquitinated Rh1, and the key autophagic component, ATG8 (LC3), Ref(2)P/P62 likely recognizes and binds all ubiquitinated proteins on the ER rather than specifically interacting with Rh1. Moreover, the antibody against Rh1-RFP only poorly recognized the ubiquitinated form. It is therefore hard to detect the direct interaction, which does not mean that they did not interact with each other. We did not include these negative results in the manuscript.



5. Results line345 is not consistent with the data presented in Fig7g-h. USP15-3 overexpression does not seem to affect levels of wild-type Rh1 in Rh1P37H-GFP perkRNAi flies. How about the levels of Rh1ph mutant?

We are sorry for this mistake in communicating the results, and thank the reviewer for pointing this out. We have corrected this sentence to "To further test this hypothesis, we overexpressed the general cytosolic deubiquitinase, USP15-31, in *Rh1^{P37H}-GFP perk^{RNAi}* flies and found that USP15-31 restored levels of wild-type Rh1 in *Rh1^{P37H}-GFP perk^{RNAi}* flies without affecting the levels of Rh1^{P37H}-GFP (Figures 7G and 7H)." (Line 380-383)

Minor
MW should be included in all western blots.

We have added MW indicators to all western blots as suggested. Please see figures for details.

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript, the authors establish many useful new tools to interrogate the role of various ER stress signaling branches in Drosophila models of human eye diseases including autosomal dominant retinitis pigmentosa. In a mutant rhodopsin-induced degradation of wild-type rhodopsin eye model, a large-scale chemical genetic screen shows that PERK-eIF2a signaling is necessary to maintain rhodopsin homeostasis. Loss

of PERK prevents proteasomal degradation of mutant rhodopsin, which leads to autophagic degradation of wild type rhodopsin. I think the story is generally interesting and important, but some of the claims may be overinterpretations, some of the data are inconsistent and I would like to see some further developments (please see below) before recommending this work for publication. Also, English language usage must be improved.

1. As for PERK loss-induced changes on a Rh1 P37H background: p62 is upregulated and colocalizes with both wt mutant Rh1, while autophagy is induced and Atg8a colocalizes with wt but not mutant Rh1. P62 is a selective receptor that aggregates ubiquitinated cargo, so I miss testing where the ubiquitinated cargo is found that "recruits" p62: on the surface of specific subdomains of the ER? Everywhere on the surface of ER? Please also discuss how it may get there - e.g., ubiquitinated proteins get stuck while being transported out of the ER?

Blocking PERK signaling resulted in the accumulation of ubiquitinated proteins and the induction of autophagy components, including P62. This correlated with the findings that proteins in the ER underwent autophagic degradation. As a selective receptor that binds ubiquitinated cargo, we speculated that ubiquitinated proteins on the ER surface accumulated. To test this, we extracted the membrane component and found ubiquitination of membrane proteins. In particular we found large increase in *Rh1*^{P37H}-*GFP perk*^{RNAi} flies. This increase was reversed by *ire1*^{RNAi} (Figure S4J, line 378-380). Moreover, we further measured mRNA levels of *hrd1* and *sordd1*, two ER-associated ubiquitin ligases, and found that transcriptional level of *hrd1* (but not *sordd1*) was also induced by the IRE1-XBP1 signaling pathway (Figure 5G, line 315-317). These new data and the molecular characteristics of P62 support our conclusion that autophagy of ER is induced in *perk* mutants. We added these data to the new Figure 5G and Figure S4J.

We also discuss this point in the discussion section as “As a scaffold protein, the autophagic adapter Ref(2)P/P62 delivers ubiquitinated proteins for selective autophagic degradation by: 1) interacting with LC3/ATG8 through its LIR region, and 2) interacting with ubiquitinated proteins through its ubiquitin-associated (UBA) domain (Moscat and Diaz-Meco, 2012; Nezis et al., 2008). In prolonged ER stress (both *Rh1*^{P37H}-*GFP perk*^{RNAi} and *ninaE*^{G69D} photoreceptor neurons), membrane proteins including rhodopsin get stuck and ubiquitinated in ER. Thus Ref(2)P/P62 and LC3/ATG8 proteins also accumulate, and wild-type rhodopsin and ER-GFP can be detected within Ref(2)P/P62 puncta.” (Line 499-506)

2. Most importantly, not only the level of ER-GFP (Fig S6b) but also mito-GFP (S6c) decreases in a statistically significant manner, and there is a tendency of decreasing cytosolic GFP as well (S6e - I wonder if this obvious change would also become statistically significant if more western blots are done and evaluated, as currently sample number n is only 3).

We repeated these experiments three more times (n=6), and indeed changes in mito-GFP and cytosolic GFP also became statistically significant, although to a much less extent than the reduction of ER-GFP. We think that the slight decrease in mito-GFP and cytoplasmic GFP may be due to the unhealthy state of cells under acute ER stress in combination of *Rh1^{P37H}-GFP* and *perk^{RNAi}*, as the photoreceptor cells are damaged and dysfunctioned at this stage (See the Figures S3C-E). Supporting this, levels of mito-GFP were absolutely unaffected, but the ER-GFP was significantly reduced in the *ninaE^{G69D}* model, which have normal photoreceptor function and structure at an early age (Figures S5E-S5H). We updated Figures S4B-H and modified the sentence with a possible explanation (Line 340-343). Please see the new Figure S4B-H, and corresponding results for details.

As the reviewer suggested below, we have toned down our statements regarding specific ER-phagy. Instead we mention selective autophagy for the most part.

How do we know that wild-type Rh1 is (only) in the ER when it is captured by autophagy? Direct evidence for its selective ER-phagy is lacking. While I'm fine with the claim that PERK loss induces (general?) autophagy in Rh1 mutant expressing cells that leads to a decline in wild-type Rh1 level, I suggest toning down a bit the involvement of a specific ER-phagy pathway (including the abstract, say, "induction of autophagy including ER-phagy") unless more convincing further data can be added to support its ER selectivity and exclusivity.

We do have cell biological evidence that autophagy of proteins in the ER compartment could be strongly detected in both *Rh1^{P37H}-GFP perk* and *ninaE^{G69D}* models. Using the *ninaE^{G69D}* model, we co-stained for Ref(2)P/P62 together with ER-GFP, mito-GFP, or cytosolic GFP. Only ER-GFP colocalized with Ref(2)P/P62, indicating that wild-type Rh1 is in the ER when it is captured by autophagy. In *Rh1^{P37H}-GFP perk^{RNAi}* flies, Rh1 was detected in cytosolic puncta that colocalized with Ref(2)P/P62 and Atg8a. Supporting these staining results, we also provided biochemical evidence that in both *Rh1^{P37H}-GFP perk^{RNAi}* and *ninaE^{G69D}* photoreceptor cells, levels of ER-GFP and wild-type rhodopsin were dramatic reduced, whereas proteins in other cellular compartments (mito-GFP and cytosolic GFP) were less affected.

As suggested, we further found that two known ER-phagy receptors (*trp1/sec62* and *atl/atl3*) are also induced in *Rh1^{P37H}-GFP perk^{RNAi}* flies. This can be reversed by knocking down *ire1.* By contrast, transcription levels of two mito-phagy receptors (*nipsnap* and *phb2*) were not regulated by the IRE1-XBP1 pathway. These data further suggest that the induction of autophagy, especially autophagy of ER, was induced under prolonged ER stress. We added these results to Figure S4I and a description has been added to the results section (Line 343-349).

Although the evidence is strong, as the reviewer points out, levels of mito-GFP and

cytosolic GFP were also reduced in *Rh1*^{P37H}*-GFP perk*^{RNAi} flies. We cannot exclude the possibility that non-ER proteins were in the autophagosome. Therefore, we have "toned down a bit the involvement of a specific ER-phagy pathway." Please see the updated manuscript for details.

3. Transcriptional upregulation of a few selected Atg genes is shown in fig 5f, but some of the most important ones are missing: Atg9 and Atg8a are rate-limiting for autophagosome initiation/numbers and size, respectively, and a number of papers showed that Atg8 family genes are usually the most highly upregulated ones not only in yeast but also in Drosophila. Please also test these two genes/mRNA levels in your setting.

As suggested, we measured mRNA levels of *atg8a* and *atg9* and found that they were both induced in *Rh1*^{P37H}*-GFP perk*^{RNAi} flies. Knocking down *ire1* reversed these inductions. We included these data in updated Figure 5F. Please see the updated Fig. 5F and corresponding results section for details (Line 312-315).

4. Fig S6a: arrows point to "empty-looking" vesicles, which are unlikely to be autophagosomes. Autophagosomes have two limiting membranes and contain cytoplasm, including organelles such as ER fragments. Please provide high-mag images of autophagosomes that fulfil these criteria or refrain from calling these vesicles autophagosomes.

We thank the reviewer for pointing this out. We realize that the previous image may have been misleading. Now we provide high-mag images of autophagosomes. We moved these results to Figure S4A. Please see the image for details.

5. It would be interesting to test the effect of p62 loss on the ninaE model phenotypes - is it similar to its effects in the Rh1 mutant model?

To test the effect of *p62* loss in the *ninaE* model, we used the CRISPR-CAS9 system to generate a *ref(2)P/p62* null mutant fly (*ref(2)P*^{m}) by deleting 4 nt within the coding region, presumably causing a frame-shift, (See the method and supplemental Figure S5I). The *ref(2)P*^{m} flies itself did not exhibit dysregulated autophagy in photoreceptor cells. In the *ninaE*^{G69D} model, the autophagy marker Atg8a was massively induced and accumulated in puncta. However, when *ref(2)P/p62* was knocked out in the *ninaE*^{G69D} model, the formation of Atg8a puncta was largely abolished. Moreover, as we did for the *ninaE*^{G69D} model, we stained for Atg8a in *Rh1*^{P37H}*-GFP perk*^{RNAi} *ref(2)P*^{RNAi} retina and found that the number of Atg8a puncta was also dramatically decreased compared with *Rh1*^{P37H}*-GFP perk*^{RNAi} flies. These results strongly support that the massive autophagy in both *ninaE*^{G69D} and *Rh1*^{P37H}*-GFP* models are P62 dependent, and indicate that this type of autophagy is selective, especially for ER components. These data were added to Figures S5I-J and 6C, and descriptions have been added to the results section (Figure S5I-J, Line 402-407) (Figure 6C, Line 332-335).

Also, why is the loss of p62 rescuing (fig 6e) - is it the loss of ubiquitinated protein aggregation, the loss of their autophagy, or both?

We stained for Atg8a in *Rh1^{P37H}-GFP perk^{RNAi} ref(2)P^{RNAi}* flies and found that the number of Atg8a puncta was also dramatically decreased compared with *Rh1^{P37H}-GFP perk^{RNAi}* flies. However, the aggregation pattern of Rh1 was not affected. These observation suggest that loss of Ref(2)P/P62 occurs downstream of rhodopsin aggregation and/or ubiquitination. These data were added to Figure 6C, and discussed in Line 332-335.

Published flies having CRISPR mutant p62 that cannot bind Atg8 to deliver cargo for autophagy could be used to test this (PMID: 35184662), or at least discuss this please.

We also noticed this paper, and now we cite and discuss it in the revised manuscript on Page27 (Line 512-516), as "Our results suggested that tuning down selective autophagy may protect against retinal degeneration. Interestingly, it has recently been reported that disrupting the interaction between Ref(2)P/P62 and Atg8a increases the tolerance to oxidative stress and reduces levels of aging-associated mitochondrial superoxide in *Drosophila* (Bhattacharjee et al., 2022)".

Reference:
Bhattacharjee, A., A. Urmosi, A. Jipa, L. Kovacs, P. Deak, A. Szabo, and G. Juhasz. 2022. Loss of ubiquitinated protein autophagy is compensated by persistent cnc/NFE2L2/Nrf2 antioxidant responses. *Autophagy*. 18:2385-2396

Minor

6. I don't see p62 puncta in ninaE mutant cells (fig 8c), which is at odds with the statistics (8d). Please clarify.

We have provided a different image that was used for the previous statistical comparison. The Ref(2)P/P62 puncta are more obvious in this image. Please see updated Figure 9C.

7. While p62 upregulation in perk RNAi is really convincing in immunostainings (fig 5 d,e), differences seem much smaller on the current wb/fig 5b then the statistics/fig 5c. Please use a more representative blot.

We have repeated the western blot in Fig. 5b 3 more times and recalculated the statistics in Fig. 5C (n=6). The results and conclusion are unchanged. Please see the updated Figure 5 for details.

8. The authors summarize their findings at the end of the intro, in 16 lines. I think it's too long for an introduction chapter.

We shorten the last paragraph of the introduction.

9. There are studies showing that PERK/eIF2a induces autophagy in Drosophila and mammalian cells while autophagy is induced by loss of PERK and accumulation of mutant rhodopsin in the present model, with the IRE1/XBP1 signaling being important (although it's not clear how). Please elaborate a bit on this in the discussion.

Yes, we thank the reviewer for this suggestion. We now discuss this issue in the discussion section on Page 25 (Line481-483), as “Studies in both *Drosophila* and mammalian cells have demonstrated that induction of PERK/eIF2α induces autophagy (B'Chir et al., 2013; Nagy et al., 2013), indicating that PERK/eIF2α signaling could regulate autophagy through different signaling pathways”.

Reference:

B'Chir, W., A.C. Maurin, V. Carraro, J. Averous, C. Jousse, Y. Muranishi, L. Parry, G. Stepien, P. Fafournoux, and A. Bruhat. 2013. The eIF2α/ATF4 pathway is essential for stress-induced autophagy gene expression. *Nucleic Acids Res*. 41:7683-7699.

Nagy, P., A. Varga, K. Pircs, K. Hegedus, and G. Juhasz. 2013. Myc-driven overgrowth requires unfolded protein response-mediated induction of autophagy and antioxidant responses in Drosophila melanogaster. *PLoS Genet*. 9:e1003664.

10. Based on the Methods section, normality of data distribution was not tested. Please evaluate the normality of all datasets, because different statistical tests must be used for ones that have non-Gaussian distribution (e.g., u test instead of t test in case of pairwise comparisons).

We have evaluated the normality of all datasets using a graphical normality test based on histograms, and all data for statistical tests are normally distributed. We explain this in the methods section (Line 829-832).

11. English language usage must be improved. I list here a couple of mistakes from the first part as examples:

L51 which induces targets the...

We have changed this sentence to “which targets and splices mRNA encoding the transcription factor X-box-binding protein 1 (XBP1), thereby activating it” (Line 63-64).

L58 it is not clear to me what „opposite activating states" means

We have changed this sentence to “The three UPR pathways, in particularly the IRE1 and PERK branches, have different activating states, and unequal or contradictory effects on cellular pathophysiology depending on the disease and physiological context” (Line 69-71).

L97 manor

We have changed “manor” to “manner” (Line 110).

L102 and whether ER-phagy to maintain cellular

We have changed this sentence to “It remains unclear how the UPR induces autophagy, and whether this form of selective autophagy is responsible for maintaining cellular homeostasis” (Line 114-116).

L118 to clear proteins on the ER

We have deleted this sentence in the updated version.

And so on...

We thank the reviewer for pointed out these mistakes. In addition to these changes, we have carefully checked the manuscript several times, and corrected the mistakes we found.

February 6, 2023

RE: JCB Manuscript #202208147R

Dr. Tao Wang
National Institute of Biological Sciences
No. 7, Park Road, Zhongguancun Life Science Park, Changping District,
Beijing 102206
China

Dear Dr. Wang:

Thank you for submitting your revised manuscript entitled "PERK prevents rhodopsin degradation and retinal degeneration by inhibiting IRE1-induced autophagy". We would be happy to publish your paper in JCB pending final revisions sought by reviewers and changes to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.

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2) Figures limits: Articles may have up to 10 main figures and 5 supplemental figures/tables.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.
** We feel both the abstract and the title may cause confusion to readers, so we suggest making the following changes:

First, the title should mention autosomal dominant retinitis pigmentosa (adRP). This could be accomplished by changing "and" to "during": "PERK prevents rhodopsin degradation during retinal degeneration by inhibiting IRE1-induced autophagy"

Next, the abstract describes "a selective form of autophagy" but does not state that this is autophagy of the ER which was shown in this revision. Third, "proteasomal activities" in the abstract should be changed to "proteasome activity". Finally, the last sentence in the abstract asserts "a physiological role for autophagy in this neurodegenerative condition" when this is a pathological role.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

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14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Hong Zhang
Monitoring Editor
Journal of Cell Biology

Tim Fessenden
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Using a Drosophila model of autosomal dominant Retinitis Pigmentsa, here, the authors delineate the roles of the IRE1-XBP1 and the PERK branches of the Unfolded Protein Response. They show that PERK plays a protective role in part by reducing mutant Rho proteins and suppressing IRE1-XBP1 signaling. Loss of PERK induces autophagy (through the IRE1-XBP1 branch) to specifically degrade the wild type Rho protein, and such conditions accelerate retinal degeneration.

All the points that I had raised during the initial round of review have been adequately addressed by the authors.

Reviewer #2 (Comments to the Authors (Required)):

The authors have addressed all my comments and I recommend it for publication.

Reviewer #3 (Comments to the Authors (Required)):

The authors have properly addressed my previous comments, so I recommend this work for publication - after some remaining issues are dealt with:

1. page 18, trp1 and atl are claimed to be ERphagy receptors while nipsnap and phb2 are claimed to be mitophagy receptors.

However, as far as I know, these fly proteins (uncharacterized as receptors) are the homologs of the corresponding human proteins (whose such roles was investigated). First, please cite references showing that these proteins are involved in ERphagy and mitophagy, respectively. Second, if no data is available for these Drosophila proteins, then refrain from calling them selective receptors (unless data can be included in this manuscript). Instead, these should be referred to as Drosophila homologs of selective receptors functioning in human cells. The same goes for the legend of Fig S4.

2. I strongly disagree with calling the new Ref(2)P mutant as "null" (line 404) because the frameshift only affects sequences after about aa225. As such, a truncated protein still containing the PB1 and ZZ domains is very likely expressed, similar to the previously described od3 allele (see Nezis 2008). This protein fragment is clearly unable to function as a selective autophagy receptor (with the LIR sequence and UBA domain missing), but PB1- and ZZ-mediated protein interactions are likely maintained. Please change the text accordingly.