

Summary of revisions

We thank the reviewers for their very careful evaluation of our manuscript. We took into consideration their comments, which we found all relevant. Specifically, the reviewers suggested to perform complementary analyses and controls, including more appropriate statistical analyses than a systematic use of t-test. To address Reviewer 2's major concern, we added new data: we analyzed another mutant of the p38MAPK pathway, the *sek-1* MAPKKK mutant. Performing these analyses further strengthened our conclusion that the p38MAPK pathway is required in PHA neurons for sensitivity to low doses of H₂O₂ (see new Fig 4, panels D-F). Furthermore, we systematically re-did all statistical analyses taking into account the multiple comparisons problem. Importantly, performing these more stringent statistical analyses did not change our conclusions.

For these reasons, we think that our revised manuscript has significantly improved compared to its initial version, and we believe that it fits PLOS ONE's publication criteria.

Figures have been revised as follows:

- Figure 1 : panels E-F have been updated and include more data points, to reinforce the point that an increased PRDX-2::GFP signal is only observed in the gut upon a 10mM H₂O₂ treatment.
- Figure 2: panels C-D have been updated (multiple comparison statistical tests) and panel E has been transferred to a supplementary figure (S3 Fig), as it contained data retrieved from WormBase (addressing Reviewer 1's point 7).
- Figure 3: panels H and L have been updated with the multiple comparison statistical tests, and their legends have been modified accordingly (addressing R1 point 3); panel K has been updated with 2 additional movies.
- Figure 4: panels D-F include the new data obtained with *sek-1* MAPKK mutants analyses (addressing R2 point 1) and revised statistical analyses (R1 point 3).
- Figure 5: the hypothetical models from panels A-B have been moved to a supplementary figure (S12 Fig) to address R1 point 8.

Supplementary Figures have been revised as follows:

- S1 Fig includes new panels (F-H) to ascertain the identity of PRDX-2-expressing PHA/PHB neurons (addressing R1 point 1)
- S2 Fig includes a new panel (C) corresponding to the quantification of the gut autofluorescence in control animals treated with H₂O₂ (addressing R1 point 4), demonstrating the specificity of the PRDX-2::GFP signal.
- S3 Fig has been added, corresponding to the former bottom panel of Figure 2, showing the presumptive transcription factors binding sites in the promoter of *prdx-2* (addressing R1 point 7).
- S10 Fig has been added, showing the individual fluorescence intensity measurements of the new supplementary *sek-1* movies, and illustrates the lack of response of *sek-1* mutants to 10μM H₂O₂, as observed in *pmk-1* mutants (addressing R2 point 1).

- S13 Fig has been added, corresponding to the former top panel of Figure 5 (addressing R1 point 8). It also includes a new panel (A) with an alignment between GUR-3 and LITE-1 proteins, showing their conserved intracellular cysteines, further reinforcing the presumptive receptor activation model proposed in the discussion.

Supplemental material has been added, to support the new data included in the revised version (especially Figure 4):

- movie 16 (related to S10 Fig) - *sek-1* mutant I2 response to 1mM H₂O₂
- movie 17 (related to S10 Fig) - *sek-1* mutant PHA response to 1mM H₂O₂
- movie 18 (related to S10 Fig) - *sek-1* mutant I2 response to 10μM H₂O₂
- movie 19 (related to S10 Fig) - *sek-1* mutant PHA response to 10μM H₂O₂

S1-S3 supplementary Tables have been reorganized to address R1 point 6.

Concerning the References list, note that we have included 2 new papers: Zhang et al., 2022 (ref 62), which came out while we were working on revisions. It has been cited in the Discussion section (p. 11) for the reasons explained below (see R2 point 2 response). We have also added Quintin and Charvin (ref 73) in the Result section (p. 6), which has been submitted in the meantime and is currently under revision at micropublication Biology. This study, which describes a left-right asymmetry in I2 and PHA neurons, justified our approach to analyze individually left and right I2 and PHA neurons.

Please find below the original reviews as well as our reply/ comments in bold.

Reviewer #1: The work of Quintin et al. examines the role of hydrogen peroxide sensing at the head and tail of *C. elegans*. The I2 pharyngeal neurons in the head and phasmid PHA neurons in the tail can both respond to H₂O₂, but with different sensitivities. The PHA neurons are more sensitive to micromolar concentrations whereas the I2 neurons require millimolar levels. The peroxiredoxin PRDX-2 is required in both cases, but may regulate detoxification in the I2 neurons and ROS signaling in the PHA neurons. The receptors GUR-3 and LITE-1 were found to play a role in light sensing and hydrogen peroxide sensing in the I2 neurons and PHA neurons, respectively. The paper provides insight into how animals can respond to the same signal through different mechanisms. Overall the experiments add to the field but there are some areas for improvement.

We thank R1 for his globally positive evaluation of our work, and for his/her suggestions for improvement. However, we were a bit puzzled to read that R1 wrote that PRDX-2 ‘may regulate detoxification in the I2 neurons and ROS signaling in the PHA neurons’. We understand from this comment that our message was not fully clear, as we clearly think that PRDX-2 is involved in ROS signaling also in I2 neurons. We have tried to make this more clear in this revised version (see Results, page 5 and Discussion, page 9).

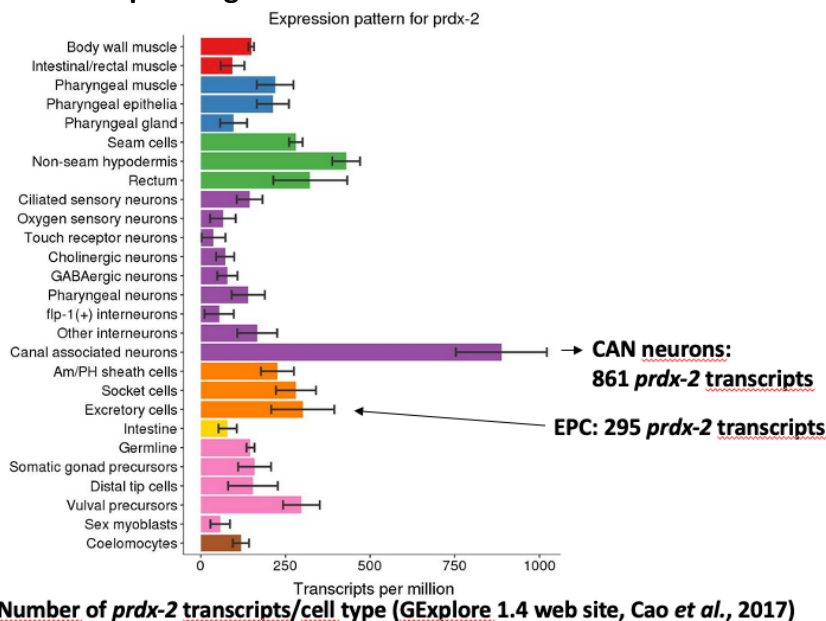
Point 1- The authors measure changes in *prdx-2::GFP* expression in response to H₂O₂ in different cell types. How did the authors confirm the localization/identity of the neurons?

We agree with R1 that neuron identification can sometimes be difficult in *C. elegans*. However, here, it was not the case for the reasons detailed below.

Regarding I2 neurons: as stated in the manuscript (page 4), previous work already reported the expression pattern of a PRDX-2 reporter transgene, especially in I2 neurons (Isermann *et al.*, 2004 ; Bhatla and Horvitz, 2015). Importantly, a functional validation of this finding has been performed by mutant rescue analyses (Bhatla and Horvitz, 2015). The authors demonstrated that the light-induced feeding inhibition defect of *gur-3* and *prdx-2* mutants are both rescued by specifically restoring *gur-3* and *prdx-2* functions in I2 neurons, using an I2-specific promoter (termed “I2::*gur-3*” and “I2::*prdx-2*”), derived from the *flp-15* gene (Kim and Li, 2004). Therefore, as GUR-3 and PRDX-2 functions are required in I2 neurons, there is no doubt on the identity of these head neurons.

Regarding CAN and PHA/PHB neurons; we indeed report for the first time expression of PRDX-2 in CAN and PHA/PHB tail neurons. The first assumptions were made by comparing the localization of PRDX-2::GFP positive neurons with neurons maps available at Wormatlas.org, as explained below.

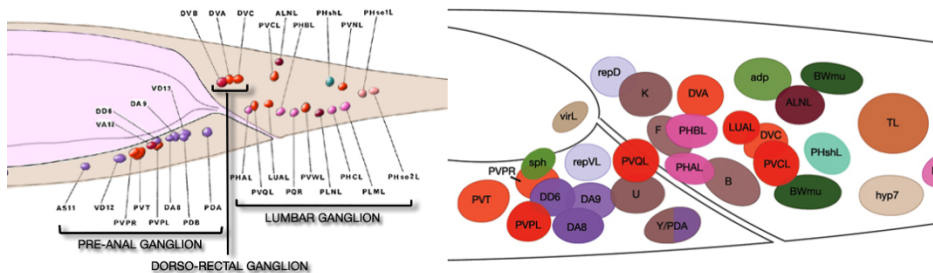
- CAN neurons were easy to identify, as they are the only neurons positioned on each side midway along the body, labeled by PRDX-2::GFP (see Fig 1). Two lines of evidence support this observation (shown below): *prdx-2* transcripts were abundantly detected in CAN neurons (Cao *et al.* 2017), and CAN neurons exhibit a very strong oxidized/reduced HyPer signal following exposure to H₂O₂ (Back *et al.*, 2012), suggesting that these neurons are very sensitive to H₂O₂, as expected for PRDX-2 expressing cells.



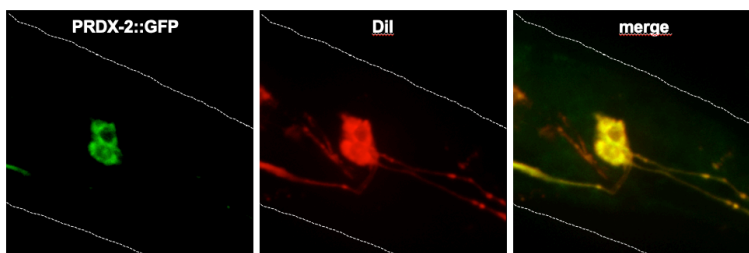


This is indicated in the manuscript (p. 4): “Consistent with this, a high number of *prdx-2* transcripts was detected in CANs and in the EPC (Cao et al, 2017).

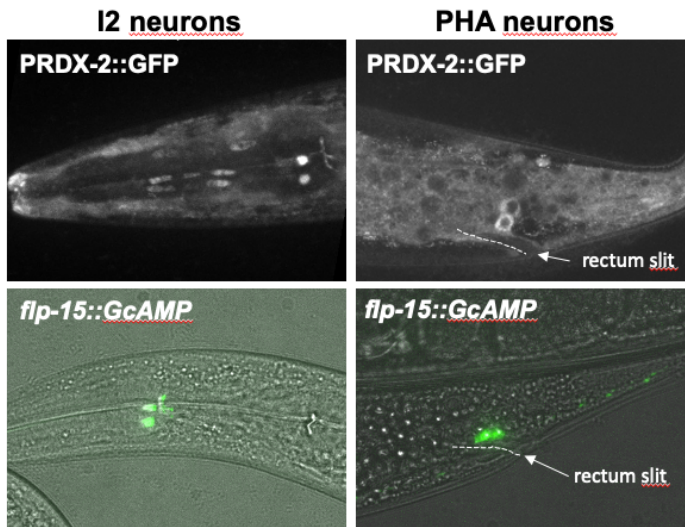
- **Regarding PHA/PHB neurons:** their identification was made easier due to the fact that there are much fewer neurons in the tail than in the head. Based our initial observations, PRDX-2::GFP candidate neurons could have been DVA, DVB or DVC from the dorso-rectal ganglion, or anterior-most neurons from the lumbar ganglion, ie PHA, PHB (named phasmids) or PVQ, as illustrated below (Wormatlas images). Due to the rather posterior and ventral position of PRDX-2::GFP neurons right above the rectum slit, dorso-rectal ganglion neurons were excluded.



To discriminate between the remaining candidates (phasmids vs PVQ interneurons), a Dil filling assay was done in the PRDX-2::GFP knock-in line (a common method in *C. elegans* to stain amphid and phasmid sensory neurons, which are exposed to the environment). As PRDX-2::GFP neurons were stained by the red lipophilic dye, this validated the neuronal PHA/PHB identity (see below, we added this data to S1 Fig).



As a last piece of evidence, the GcAMP reporter we used, driven by the *flp-15* promoter (specific to I2 and PHA neurons, Kim and Li, 2004) marks the same cells as PRDX-2::GFP (shown below).



In conclusion, we are certain about the identity of PRDX-2::GFP-expressing neurons, however we preferred not to include such a level of detail in the supplementary data as they already comprise 13 figures and 24 movies. As referenced on page 4, we included a new panel in S1 Fig (F-H), showing the Dil staining.

Point 2- The concentrations of H₂O₂ differ experiments and range from 10mM, 1mM and 10uM. Please provide rationale for changing the concentrations in each experiment. Also, a discussion on the biological relevance of these concentrations would be helpful for the reader.

We agree with R1 that this point deserved a clarification.

The choice of H₂O₂ concentrations was based on previous work, describing the animal's response to various doses of H₂O₂. A sentence has been added in the manuscript (p.4) : 'We selected H₂O₂ concentrations inducing different physiological responses (Bhatla and Horvitz, 2015). Interestingly, our observations regarding PRDX-2::GFP higher expression in the gut upon a 10mM treatment can find an explanation in the different behavior reported, as discussed later in the same paragraph (see p. 5, top).

Regarding neuron response analyses, we chose 1mM and 10μM again on the basis of the difference reported in animals' physiological response: whereas at 1mM H₂O₂ nearly all animals stop pharyngeal pumping, the 10μM dose induces a less penetrant response, which we precisely aimed to test. We modified the sentence on p. 6 as follows:

While it was initially stated: 'We thus tested whether I2 and PHA neurons exhibit a response to the very mild dose of 10μM H₂O₂. Indeed, at this dose, it has been reported that only a minority of animals (≈ 35%) respond by inhibiting pharyngeal pumping (Bhatla and Horvitz, 2015)' ; **the sentence was changed as follows:** 'We thus tested whether I2 and PHA neurons exhibit a response to the very mild dose of 10μM H₂O₂, a dose which induces a less penetrant pharyngeal pumping inhibition (≈ 35% of animals) than that observed at 1mM (≈ 90% of animals, Bhatla and Horvitz, 2015).

Point 3- Statistical analysis should be improved. For some figures, a t-test is not appropriate.

We thank again R1 for the major improvement suggested here. For pairwise comparisons of data sets with a normal distribution (or N>30), p-values were calculated using an unpaired two-tailed Student test, and the Welch correction was applied when samples variance was not homogeneous. When distributions were not normal (Anderson-Darling and Shapiro Wilk tests not satisfied), a Mann Whitney test was used. Multiple comparisons were analyzed using a one-way ANOVA with Bonferroni's correction (for normal distributions), or a non-parametrical Kruskal-Wallis test followed by a Dunn's multiple comparisons test. Importantly, performing these new statistical analyses on our data did not change the validity of our conclusions. These modifications have been indicated in the paragraph describing statistical analyses (page 15) and in Figure legends, accordingly.

Welsh's t-test should be corrected to "Welch"
Done.

Point 4- Figure 1 – The authors show an increase in signal following exposure to H₂O₂ in the intestinal cells, which the authors attribute to increased expression of prdx-2::GFP. However, it appears that the time hours of 1-2 hours may not be sufficient to induce protein translation and the signaling could be an artifact of gut granule autofluorescence in response to the H₂O₂. The authors should demonstrate the selectivity of the single for prdx-2. In addition, the supplemental data in WT control should be quantified (2A and B).

Regarding the possibility that PRDX-2::GFP could respond later than the examined time point, in our initial experiments we did not observe any significant difference in PRDX-2::GFP level when animals were analyzed 3-4 hours vs 1-2 hours after H₂O₂ treatment. Notably, in yeast, the peroxiredoxin reporter Tsa1-GFP (equivalent of PRDX-2) shows rapid induction with a peak within the 100 first minutes following the H₂O₂ stress (Goulev et al., 2017). To demonstrate the specificity of the PRDX-2 GFP signal, we are providing a quantification of H₂O₂-treated controls, which do not exhibit higher gut granule fluorescence after 10mM H₂O₂ treatment, in contrast to PRDX-2::GFP animals. This control quantification panel has been added in the revised version (S2 Fig, panel C). We are grateful to R1 for suggesting this improvement, which strengthens our conclusions.

Point 5- From Figure 1, the authors make conclusions on the role of prdx-2 in I2 neurons based on expression changes (or lack thereof). While I agree that the responses of prdx-2 to oxidative stress are most likely "cellular context dependent", the author's conclusion is solely based on a GFP signal, which does not report on prdx-2 activity. Thus the authors should not overstate their conclusion.

We agree with R1 that this conclusion should not be overstated, relative to the actual experiments. We indicated this limitation in our paragraph conclusion in the

following manner (p. 5):

‘Although the induction we observed is only based on expression level and not on protein activity, we suggest that PRDX-2 could still scavenge H₂O₂ in the EPC and in the foregut, consistent with the reported protective role of intestinal PRDX-2 (Oláhová et al., 2008).’

Point 6- The article has grammatical issues and unfinished sentences. As a note, the supplemental tables are not represented as tables. Genomic nomenclature should be followed throughout – sometimes the gene is listed when referring to the protein and vice versa. Also some of the titles do not fit with the data. One example, “Expression pattern of prdx-2 and its evolution upon H₂O₂ treatment.” How is prdx-2 evolving with H₂O₂ exposure? I would recommend editing throughout.

We thank again R1 for helping us to improve the quality of the writing. We have changed the paragraph title into : ‘Expression pattern of PRDX-2::GFP and its variation upon H₂O₂ treatment’

Genomic nomenclature has been addressed as it should be in the *C. elegans* field, ie with gene names in small letters and proteins in capital letters (this does not apply to yeast Yap1). We modified the supplemental material so that only true tables are named tables (ie Tables S1-S3), while strain and primers lists are no longer called Tables but just ‘lists’. In addition, the revised manuscript has been extensively proof-read.

Point 7- Figure 2 – The wormbase analysis seems more suited for the supplement since this is not new data. Moreover, it is unclear if the authors are permitted to reuse the data in their figure.

We agree with R1 that this figure panel was more appropriate in a supplementary figure, as it was not based on our own findings. As commonly practiced in the literature related to *C. elegans*, we incorporated a screenshot arising from our data mining of Wormbase, properly citing the people who generated the raw data (in this case, Niu et al, 2011) and acknowledging WormBase (p.16). The panel has been moved to S3 Fig.

Point 8- Figure 5 – The hypothetical model provided in C and D are helpful and supported by the data provided. However, A and B do not add any value and are confusing since the authors did not test or discuss any specific cysteine residues in their protein target. Additionally, it appears that the cysteine residues do not match with the protein sequences provided on wormbase.

We appreciated R1’s positive evaluation of our model (formerly Fig 5CD). Regarding the presumptive PRDX-2-mediated receptor activation (formerly Fig 5AB), we agree with R1 that the model is speculative, and has not been tested experimentally. Still, we think that it has conceptual value, as it was inspired by an important literature survey and discussions with experts in the yeast Redox community (especially Dr Jacquel, acknowledged p. 16).

The PRDX-2 activation model has therefore been moved to a new supplementary figure (S12 Fig). In addition, we included a protein alignment between GUR-3 and LITE-1 receptors (panel A), to illustrate that conserved cysteines are only present in intracellular or transmembrane domains, thus supporting a presumptive intracellular activation by

PRDX-2. Cysteine numbering has been corrected —we are grateful to R1 for noticing such a detail.

Point 9- The discussion is very detailed and often meanders from discussing the findings of the manuscript. I would suggest condensing the discussion to avoid over interpretation.

We condensed the discussion as much as we could, and did our best to avoid over interpretation. We especially attempted to place our interpretations in the context of a meaningful review of the relevant literature, also benefiting from the input of our Redox specialist colleagues. Thus, we feel that many of our discussion points are relevant and fill some gaps in the field. Therefore, we hope to have reached a satisfactory level of concision, while still highlighting biological connections that are important to understand this biological system.

Reviewer #2: The manuscript submitted by Quintin et al identified how oxidants such as hydrogen peroxide (H₂O₂) are perceived by the I2 pharyngeal neurons and the tail phasmid PHA neurons in *C. elegans*. Using a GFP knock-in line for the peroxiredoxin PRDX-2, the authors demonstrate this peroxiredoxin functions as a putative H₂O₂ sensor in both I2 and PHA neurons, while in the anterior gut and excretory pore cell it functions mainly as a peroxidase. Furthermore, the activation of the I2 and PHA neurons were dependent on the concentration of H₂O₂ administered which led to the identification of distinct molecular mechanisms to explain differences in sensitivity. Gustatory G-protein-coupled receptors GUR-3 and LITE-1 were shown to be expressed and required for the perception of H₂O₂ in the I2 and PHA neurons respectively. Further investigation using microfluidics and calcium imaging revealed PHA neurons required the p38 MAPK pathway in sensing H₂O₂ which has been shown to activate the voltage gated calcium channel OSM-9 in ASH neurons in the worm. Finally, in addition to sensing H₂O₂ the PHA neurons exhibited a slow photo-response to light, while the I2 neurons exhibited a fast response. The photo-response is dependent on PRDX-2 in the I2 neurons. However, in the PHA neurons PRDX-2 is not required. The manuscript is well written and addresses an important question in the field.

We sincerely thank R2 for his/her very positive review.

Concerns

1. It is thought that dimerized peroxiredoxin can interact with ASK-1 (NSY-1) to activate the p38 MAPK. Using calcium imaging the authors show *pmk-1* is required for the sensing of H₂O₂ and suggest the p38 MAPK pathway is involved in this mechanism. However, the *sek-1* and the *nsy-1* mutant strains weren't included in the study. Including calcium measurements in the *sek-1* and *nsy-1* mutant strains would further support this claim.

We agree with R2 that our initial conclusion on the p38MAPK pathway requirement in PHA hypersensitivity to H₂O₂ relied solely on the analysis of a single mutant, the *pmk-1*/MAPK mutant. As suggested, we further assessed the involvement of this pathway in

PHA neurons by introducing the GcAMP calcium sensor in a *sek-1*/MAPKK mutant background (SXB70 new strain) to monitor I2 and PHA responses to H₂O₂. We observed that I2 neurons in *sek-1* mutants responded normally to 1mM (movie 16), but not to 10μM H₂O₂ (movie 18), as in controls and as in *pmk-1* mutants. Similarly, *sek-1* mutants PHA neurons responded normally to 1mM H₂O₂ (movie 17), while *pmk-1* mutant showed a slight diminution compared to WT. Importantly, in *sek-1* mutants, as in *pmk-1* mutants, PHA neurons were no longer able to respond to the low dose of 10μM H₂O₂ (movie 19), unlike in controls. Thus, our new analyses reveal that *sek-1* mutants almost completely phenocopied *pmk-1* mutants in their response to H₂O₂, further supporting the point that the p38MAPK pathway seems indeed to be specifically involved in PHA hypersensitivity to H₂O₂.

These new results have been incorporated in the revised version of Figure 4 (panels D-F); and they are supported by the addition of supplementary material (movies 16-19 and S10 Fig, depicting their quantification). The text has been accordingly modified in the manuscript in the results sections (paragraph now entitled '*PMK-1 and SEK-1 are required for PHA neurons response to micromolar H₂O₂ but are dispensable in I2 neurons*', p. 7-8) and in the discussion (p. 9). Given that the *C. elegans* p38-MAPK pathway is linear (Inoue et al., 2005), and that *sek-1* mutants showed the same defects as PMK-1 in PHA neurons, we considered that testing additional components of the same pathway (such as the *nsy-1* MAPKKK) was not necessary here.

2. According to the Li et al (2016) AKT-1 acts downstream of the p38 MAPK to phosphorylate OSM-9 in ASH neurons in response to H₂O₂ stimulation. Does Akt-1 act downstream of PMK-1 to activate OSM-9 in the PHA neurons? This could be tested using calcium imaging or speculated in the model. Furthermore, SKN-1 is also known to act downstream of the p38 MAPK. Could SKN-1 be a potential downstream activator if AKT-1 is not required in H₂O₂ sensing?

We thank R2 for raising this hypothesis, highlighting his/her interest in our work. Li et al. (2016) indeed provided evidence that the TRPV channel OSM-9 can be phosphorylated in vitro by the AKT-1 kinase (on OSM-T10), and that this T10 residue has a key function in vivo, in potentiating the micromolar H₂O₂-induced sensory response in ASH neurons. It is tempting to speculate that a similar mechanism could take place in PHA neurons to promote their sensitivity to micromolar H₂O₂, but we have not tested it. As suggested by R2, this has been speculated in the discussion (p. 10-11).

Interestingly, while we were working on the revisions, the same group published an article showing that DLK/p38MAPK signaling regulates LITE-1 photoreceptor's stability in ASH photosensory neurons (Zhang et al., 2022). Therefore, the involvement of PMK-1 in LITE-1 recycling in PHA neurons, as PMK-3 in ASH neurons, is another possibility. This has also been mentioned in the discussion (p. 11). All these questions should be addressed in the future, including the SKN-1 possible role proposed by R2.