Stress-induced vesicular assemblies of dual leucine zipper kinase are signaling hubs involved in kinase activation and neurodegeneration

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Dear Casper,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are included below.

As you can see the referees appreciate the findings reported, but also indicate that much further analysis would be needed to consider publication here. Should you be able to extend the findings along the lines as indicated then I would be interested in considering a revised version.

I think it would be helpful to discuss the raised points further and I am available to do so via email or video.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

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

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

I have attached a document with helpful tips on how to prepare the revised version

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (16th Mar 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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Referee #1:

The current study by Casper Hoogenraad's group at Genentech describes a systematic biochemical approach which provides insight into the mechanism of stress-activated MAP Kinase signaling in the context of trophic factor withdrawal in primary embryonic DRG neurons. The studies in this paper build upon previous work by the Genentech group (previously led by Joseph Lewcock (example papers: PMID: 23979718, PMID: 28440222, and PMID: 28993483)), and share with the previous work a robustness in technical quality. From phosphoproteomic analysis of NGF deprived eDRG cultures, the authors provide a striking finding that the dileucine zipper kinase DLK is selectively activated; this stands out from all the other MAPK signaling components whose phosphorylation state is reduced following NGF withdrawal. The study then digs further into the mechanism of DLK signaling activation and makes a few new insights. The idea that association with vesicles via regulation by palmitoylation is not new. However the authors provide some further data on this, including a finding with over-expressed DLK constructs targeted to different organelle membranes, which suggests that precise recruitment to a specific vesicle or organelle is not essential for the activation of its signaling function. These findings are consistent with previous studies that suggest that over-expression of the DLK kinase is sufficient to activate its signaling capacity (PMID: 10702297, PMID: 20921142, PMID: 19164707). The most novel (but also most tenuous) insight in the paper stems from the lipidomics approach that the authors carried out with purified DLK-associated particles from HEK cells. From this they detect a preferential association of wild type-DLK (compared to the mutant that disrupts its palmitoylation) with sphingomyelin. They then show that treatment with a
sphingomyelin synthase inhibitor D609 appears to disrupt DLK's stability and ability to associate with vesicles. Finally, the authors show that treatment of eDRGs with inhibitors of endocytosis also block DLK's vesicle association and signaling. Dynasore treatment also inhibits axonal degeneration after NGF withdrawal, an expected downstream outcome of DLK activation.

Overall, these studies provide some interesting new insights into the mechanism of pro-degenerative MAP Kinase signaling following NGF withdrawal. The mechanism of DLK signaling is poorly understood and has garnered a great deal of interest for its roles as a potential target to delay both axon loss in neuropathies and cell loss in degenerative models including glaucoma. The idea that endocytosis is required for DLK signaling activation is interesting, and raises further questions, such as whether endocytosis of a particular receptor is needed to activate DLK. In my opinion, these questions are the starting point for a follow-up story. While the current data are descriptive, they raise some new ideas, and are generally rigorous and well controlled.

I have a few suggestions and comments:

1. The lipidomics is an innovative part of the study, however the enrichment for DLK-associated lipids compared to HA-tag control is quite weak. On the other hand, treatment with the D609 inhibitor of sphingomyelin synthesis leads to a very dramatic impairment in the association of DLK with vesicles and, apparently, the total levels of DLK (shown in Figure 4K). While the authors interpret that sphingomyelin synthesis is required for the association of DLK with vesicles, the levels of cytoplasmic DLK are also dramatically reduced by the treatment. Since these effects upon DLK are so dramatic it makes one wonder about what else in the cell becomes impaired following D609 treatment. Are the cells generally sick? Some controls that show the specificity of the treatment and that other organelles that remain intact are needed to frame the interpretation of the studies with D609.

2. In Figure 5D, BFA treatment abolishes all anterograde motion in the kymographs. However this is not reflected in the quantification (Figure 5E).

3. Figure 1E uses two algorithms to identify palmitoylation sites on the different MAPKs. However the sites predicted by the two algorithms do not match and this doesn't provide much information. It can be replaced by Supplementary Figure 1D which provides evidence that multiple MAPKs are palmitoylated.

4. The quantification for Figure 2 G mentions # DLK-vesicles/μm*min as the title of the y-axis. Since the staining is from fixed images, how can the flux of DLK-vesicles be measured?

5. In Figure 3H, the number of DLK vesicles increases when DLK inhibitor is used. Why would this be the case?

Referee #2:

The manuscript entitled 'Stress-induced DLK-vesicle assembly acts as local signaling platform that drives kinase activation and neurodegeneration' by Tortosa and colleagues shows how DLK-MAPK signalling pathway is regulated by endocytosis and sphingolipids in a neuronal cell culture model upon cellular stress induced by growth factor removal. The findings are interesting and exciting, in particular, the specific localization change in DLK in this model, which is not seen in any other MAPK module in this experimental system. The authors made an excellent example how to dissect the molecular mechanism and relevance of this finding. Whilst no additional data are strictly required for completion of this study, there are a few points that merit further clarification, support by citing existing literature or a revision of wording and/or narrative.

Main points:

1. Cellular stressors are very diverse. This occurs on multiple instances: stressors are not specifically defined. The authors should be specific about which type of stressor is studied in their experiments. Are insights with this stressor/stress condition applicable to other forms of cellular stress in neurons? Eg. glutamate toxicity, excitotoxicity. Please comment and/or provide additional data. This would help drawing conclusions from the work presented in comparison with other studies.

2. The main experimental model employed in this study is in vitro NGF deprivation in DRG neurons. While this model is a valid experimental system, it is not well introduced. There is no reference, no explanation why this model was chosen in the results section when it is being introduced. Why was this work done in DRG neurons as compared with other types of primary neurons? What supports that this is a model of neurodegeneration? Can references be cited?

3. The discussion includes many interesting thoughts yet is at times somewhat detached from the actual results presented in the study. This should be revised, including a comparison with other cellular stress and animal models of neurodegeneration.
authors mention a few model studies of DLK and neurodegeneration but do not go into more detailed comparison.

Minor points:

1. Introduction: Not always clear whether the information given in the introduction applies to neurons or to other cell types. This should be made clearer, eg. by pointing out what is generalized across all cell types and what mechanisms specifically apply to neurons.

2. Fig 4 J: Is the effect of D609 treatment on DLK vesicles generalized on all vesicle trafficking (not just the ones labelled with DYLK-gfp)? Blots in 4K suggest that neurons are unaffected by this treatment. Can you comment on this in the results/discussion and/or provide additional data on the state of neurons upon D609 treatment?

3. Not all information stated by the authors referring to prior findings in the field is referenced. The text should include more citations to support the claims made/information provided.

4. p9 please rephrase sentence and logic starting with “Since dynasore...” The explanation and description of the inhibitors listed does not follow a clear rationale, because the inhibitors/treatments listed are neither more specific nor in any other way better than dynasore.

5. Fig. 6, please explain very briefly in the figure legend or in the text describing Fig 6 what the ‘degeneration index’ relates to. Please cite a relevant reference in the results section or methods. The current results from figure 6 would benefit from cell viability/death measurements to support the conclusions.

6. Fig 4B please include quantification of NGF+ with DYNasore group here.

Referee #3:

This manuscript "Stress-induced DLK-vesicle assembly acts as local signaling platform that drives kinase activation and neurodegeneration" by Elena Tortosa et al. examines the localization and function of the MAP kinase dual leucine zipper kinase (DLK) in response to neuronal stress induced by neuronal growth factor removal. The authors examine phosphorylation of a number of kinases and find that DLK is the only one examined that increases in response to NGF removal. They also show that DLK membrane association increases in response to the stressor. Previous work has shown that DLK is lipid-modified by palmitate, which is necessary for association with vesicles and downstream signaling. The authors' data largely agree with these previous findings. There are new findings, including that inhibition of internalization blocks DLK activity and that DLK needs a sphingomyelin-enriched compartment for activation. However, major flaws in the interpretation of these data need to be addressed before this manuscript is suitable for publication.

Major points:

1. The conclusion that DLK relies on endocytosis for activation is based, in large part, on use of the endocytosis inhibitor dynasore. However, dynasore is not a dynamin-specific inhibitor (Preta et al 2015). It has several major off-target mechanisms. It also interferes with cholesterol homeostasis, and cyclodextrin also disrupts normal cholesterol levels. Are the changes in DLK signaling due to alterations in cholesterol homeostasis? Other non-pharmacological approaches should be used to confirm this finding, as it is one of the main novel findings in this manuscript.

2. Another major finding was that DLK associates with a sphingomyelin-enriched compartment. The authors use immunoprecipitation with HA alone, WT DLK-HA, or non-palmitoylatable DLK-HA and then subsequent lipid analysis from the immunoprecipitated fraction. However, the authors show by immunostaining, blot, and IP that HA alone does not express in HeLa cells. In addition, non-palmitoylatable DLK has been shown to be cytoplasmic, rather than lipid-associated (Holland et al), consistent with the results in this manuscript (Figure 3E). Given these points, it is hard to understand how the authors are pulling down any vesicles at all in the HA alone or palmitoylation dead DLK conditions, let alone enough for lipid analysis. This raises serious doubts about the specificity of this assay.

3. It is not clear how the sphingomyelin synthase inhibitor D609 is working in these experiments. The authors conclude that sphingomyelin is necessary for DLK activation and downstream signaling. However, D609 seems to be also either significantly reducing (Fig 4G) or completely abolishing (Fig 4K) levels of DLK. This complicates interpretation of this experiment.

4. Kymographs are useful for following movement of vesicles, but it would be more useful to see standard images of neurons under all of the conditions. One might expect DLK to become more diffuse and cytoplasmic when not active or palmitoylated, as Holland et al reported, but it is not possible to see that in kymograph format.

5. Colocalization might be more accurately measured using an object-based colocalization, since much of the image is black.
when imaging vesicle-associated proteins.

6. Incorrect or confusing statistics are being used. For example, Figure 5B is using one-way ANOVA but changing two independent variables. Please check statistical tests throughout.

Minor points:

1. Please remove the reference to trending in the section below. If the data are not significantly different, they are not different. “The increased levels of phosphatidylethanolamine and phosphatidylcholine in the DLK fraction is trending, however only sphingomyelin levels and species were significantly different (Figure 4E, F).”

2. Figure 1E does not really add to the manuscript, since the authors are not focusing broadly on kinase palmitoylation, but specifically on DLK.
The current study by Casper Hoogenraad's group at Genentech describes a systematic biochemical approach which provides insight into the mechanism of stress-activated MAP Kinase signaling in the context of trophic factor withdrawal in primary embryonic DRG neurons. The studies in this paper build upon previous work by the Genentech group (previously led by Joseph Lewcock (example papers: PMID: 23979718, PMID: 28440222, and PMID: 28993483)), and share with the previous work a robustness in technical quality. From phosphoproteomic analysis of NGF deprived eDRG cultures, the authors provide a striking finding that the dileucine zipper kinase DLK is selectively activated; this stands out from all the other MAPK signaling components whose phosphorylation state is reduced following NGF withdrawal. The study then digs further into the mechanism of DLK signaling activation and makes a few new insights. The idea that association with vesicles via regulation by palmitoylation is not new. However, the authors provide some further data on this, including a finding with over-expressed DLK constructs targeted to different organelle membranes, which suggests that precise recruitment to a specific vesicle or organelle is not essential for the activation of its signaling function. These findings are consistent with previous studies that suggest that over-expression of the DLK kinase is sufficient to activate its signaling capacity (PMID: 10702297, PMID: 20921142, PMID: 19164707). The most novel (but also most tenuous) insight in the paper stems from the lipidomics approach that the authors carried out with purified DLK-associated particles from HEK cells. From this they detect a preferential association of wild type-DLK (compared to the mutant that disrupts its palmitoylation) with sphingomyelin. They then show that treatment with a sphingomyelin synthase inhibitor D609 appears to disrupt DLK's stability and ability to associate with vesicles. Finally, the authors show that treatment of eDRGs with inhibitors of endocytosis also block DLK's vesicle association and signaling. Dynasore treatment also inhibits axonal degeneration after NGF withdrawal, an expected downstream outcome of DLK activation.

Overall, these studies provide some interesting new insights into the mechanism of pro-degenerative MAP Kinase signaling following NGF withdrawal. The mechanism of DLK signaling is poorly understood and has garnered a great deal of interest for its roles as a potential target to delay both axon loss in neuropathies and cell loss in degenerative models including glaucoma. The idea that endocytosis is required for DLK signaling activation is interesting, and raises further questions, such as whether endocytosis of a particular receptor is needed to activate DLK. In my opinion, these questions are the starting point for a follow-up story. While the current data are descriptive, they raise some new ideas, and are generally rigorous and well controlled.

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We agree with the reviewer that the enrichment for DLK-associated lipids compared to HA-tag control is quite low. In the revised manuscript, we have improved the protocol on vesicle purification and greatly reduced the background signal (see new Figure 4E and F). Additionally, we would like to point out that D609 treatments do not reduce DLK protein levels as it is quantified in Figure 4I. Since we observe a decrease in DLK protein levels in both cytoplasmic and membrane fractionations, we have analyzed the
remaining fraction we get from our fractionation assays and have seen that DLK accumulates in this insoluble fraction under D609 treatments (see new Figure 4L). We previously included some stainings for Rab3, Rab6, Rab11 and mitochondria under D609 treatment (Appendix Figure S3D), and, in the revised version of the manuscript, we have analyzed axon integrity (Appendix Figure S3E) and performed some cell viability assays (live cell labeling and LDH assay) (Appendix Figure S3F and G). All this data together shows that cells are not drastically affected by D609 treatments at the concentration and time used in this study.

2. In Figure 5D, BFA treatment abolishes all anterograde motion in the kymographs. However, this is not reflected in the quantification (Figure 5E).

We thank the reviewer for pointing this out. In the revised manuscript we have included the quantification of the DLK- positive vesicle orientation under control and BFA treatments (new Figure 5E).

3. Figure 1E uses two algorithms to identify palmitoylation sites on the different MAPKs. However, the sites predicted by the two algorithms do not match and this doesn't provide much information. It can be replaced by Supplementary Figure 1D which provides evidence that multiple MAPKs are palmitoylated. Figure 1E has been removed from the revised version of the manuscript.

4. The quantification for Figure 2 G mentions # DLK-vesicles/μm*min as the title of the y-axis. Since the staining is from fixed images, how can the flux of DLK- vesicles be measured?

We thank the reviewer for pointing this out. We have corrected this in the revised manuscript and Y-axis are now labeled as # DLK-vesicles/μm.

5. In Figure 3H, the number of DLK vesicles increases when DLK inhibitor is used. Why would this be the case?

We thank the reviewer for raising this interesting question. We have tested DLK protein levels and they do not increase upon treatment with the DLK inhibitor. Therefore, we hypothesize that the binding of the inhibitor to DLK might help to stabilize the protein in a conformation that would favor the binding or decrease the detachment of DLK to the vesicles. However, to strengthen this hypothesis additional experiments are needed.

\[
\begin{align*}
\text{Control} & \quad \text{DLKi} \\
\text{DLK} & \quad \text{GAPDH}
\end{align*}
\]

\[
\begin{array}{c}
\text{Control} \\
\text{DLKi}
\end{array}
\]

\[
\begin{array}{c}
\text{DLK/GAPDH (AU)} \\
\text{ns}
\end{array}
\]

Figure 1. DLK inhibitor treatments do not affect DLK protein levels. Representative Western blots of DLK and GAPDH (left) and quantification of DLK protein levels (right) from 3DIV cultured embryonic DRG neurons treated with DLK inhibitor (DLKi) for 3h.

*** Referee #2 ***

The manuscript entitled 'Stress-induced DLK-vesicle assembly acts as local signaling platform that drives kinase activation and neurodegeneration' by Tortosa and colleagues shows how DLK-MAPK signalling pathway is regulated by endocytosis and sphingolipids in a neuronal cell culture model upon cellular stress induced by growth factor removal. The findings are interesting and exciting, in particular, the specific localization change in DLK in this model, which is not seen in any other MAPK module in this experimental system. The authors made an excellent example how to dissect the molecular
mechanism and relevance of this finding. Whilst no additional data are strictly required for completion of this study, there are a few points that merit further clarification, support by citing existing literature or a revision of wording and/or narrative.

Main points:

1. Cellular stressors are very diverse. This occurs on multiple instances: stressors are not specifically defined. The authors should be specific about which type of stressor is studied in their experiments. Are insights with this stressor/stress condition applicable to other forms of cellular stress in neurons? Eg. glutamate toxicity, excitotoxicity. Please comment and/or provide additional data. This would help drawing conclusions from the work presented in comparison with other studies.

We thank the reviewer for addressing this point and we more explicitly mention the stressor used in each experiment in the revised manuscript. In addition to the role of DLK in the developmental neurodegeneration model of NGF withdrawal, DLK and its downstream signaling pathway are also involved in other chronic forms of stress such as mechanical axon transection or stress associated with drugs that alter the microtubule cytoskeleton such as vincristine or colchicine. DLK also play a role in animal models of Parkinson’s disease, Alzheimer, amyotrophic lateral sclerosis, diffuse traumatic brain injury, excitotoxicity-induced degeneration, glaucoma/optic neuropathy, chemotherapy-induced peripheral neuropathy or sciatic nerve injury (Le Pichon et al, 2017; Watkins et al, 2013; Fernandes et al, 2014; Pozniak et al, 2013; Chen et al, 2008; Welsbie et al, 2017; Miller et al, 2009; Welsbie et al, 2019, 2013; Wlaschin et al, 2018; Patel et al, 2015; Hu et al, 2019; Ma et al, 2021). We agree it will be interesting to check if the mechanisms we propose here (1/ the role of sphingomyelin in controlling DLK signaling pathway and 2/ the protective effect of endocytosis inhibition in a NGF withdrawal model of neurodegeneration) are conserved in any of these other models of neurodegeneration. Since the molecular machinery that mediates neurodegeneration in the NGF withdrawal model has been shown to be reactivated in all these models, we hypothesize that the molecular mechanisms described here also play a role in other stress models different to NGF withdrawal. In fact, we observe that endocytosis inhibition is protective after vincristine exposure suggesting that the molecular mechanism defined here might be extended to other models of neurodegeneration (Fig 5K).

2. The main experimental model employed in this study is an in vitro NGF deprivation in DRG neurons. While this model is a valid experimental system, it is not well introduced. There is no reference, no explanation why this model was chosen in the results section when it is being introduced. Why was this work done in DRG neurons as compared with other types of primary neurons? What supports that this is a model of neurodegeneration? Can references be cited?

We thank the reviewer for pointing this out. A brief introduction about the in vitro NGF deprivation model, together with some references, have been added to the revised version of the manuscript (see page 5). We used this experimental model of NGF withdrawal in DRG neurons because it is a well-established and robust in vitro model of neurodegeneration in which a homogeneous population of cells initiates a process of degeneration in a synchronous and reproducible manner. Since mechanisms activated during this process of neurodegeneration have been shown to be aberrantly activated in other models of neurodegeneration such as an excitotoxicity-induced neuronal degeneration model, a chemotherapy-induced model of neurodegeneration and animal models of neurodegenerative diseases such as Parkinson’s disease, Alzheimer or amyotrophic lateral sclerosis, findings presented here might be relevant to these other models of neurodegeneration.

3. The discussion includes many interesting thoughts yet is at times somewhat detached from the actual results presented in the study. This should be revised, including a comparison with other cellular stress and animal models of neurodegeneration. The authors mention a few model studies of DLK and neurodegeneration but do not go into more detailed comparison.
We have reviewed and changed the discussion section according to the reviewer's suggestion. We have also included a paragraph mentioning more study models for DLK in the discussion section (see page 13).

Minor points:

1. Introduction: Not always clear whether the information given in the introduction applies to neurons or to other cell types. This should be made clearer, e.g. by pointing out what is generalized across all cell types and what mechanisms specifically apply to neurons.

   We thank the reviewer for mentioning this point and we now more explicitly state the cell types which are used in each experiment in the revised manuscript.

2. Fig 4 J: Is the effect of D609 treatment on DLK vesicles generalized on all vesicle trafficking (not just the ones labelled with DYLYK-gfp)? Blots in 4K suggest that neurons are unaffected by this treatment. Can you comment on this in the results/discussion and/or provide additional data on the state of neurons upon D609 treatment?

   D609 treatment doesn’t seem to affect drastically to other organelles as you can see in immunostainings performed for markers of different compartments such as exocytic vesicles, synaptic vesicles or recycling endosomes (stained with antibodies against Rab6, Rab3 and Rab11, respectively) and mitochondria (labeled with an anti-cytochrome C antibody) (Appendix Figure S3D). We have now included an analysis of the axon integrity (Appendix Figure S3E) and cell viability assays (live cell labeling and LDH assay) (Appendix Figure S3F and G) to show that neurons are viable and healthy under D609 treatments.

3. Not all information stated by the authors referring to prior findings in the field is referenced. The text should include more citations to support the claims made/information provided.

   Additional references have been added to the revised version of the manuscript.

4. p9 please rephrase sentence and logic starting with "Since dynasore..." The explanation and description of the inhibitors listed does not follow a clear rationale, because the inhibitors/treatments listed are neither more specific nor in any other way better than dynasore.

   We thank the reviewer for pointing this out. Since dynasore is a dynamin inhibitor it might affect both clathrin and caveolin dependent endocytic pathways. We used other inhibitors for each of these endocytic pathways. We have rephrased the sentence in the revised version of the manuscript.

5. Fig. 6, please explain very briefly in the figure legend or in the text describing Fig 6 what the 'degeneration index' relates to. Please cite a relevant reference in the results section or methods. The current results from figure 6 would benefit from cell viability/death measurements to support the conclusions.

   The degeneration index is based on the ratio of the areas of fragmented axons versus total axonal area. A brief explanation has been included in figure legend 6 and a relevant reference in Method section (see page 27).

6. Fig 4B please include quantification of NGF+ with DYNasore group here

   Quantifications have been added to the revised version of the manuscript.

*** Referee #3 ***

This manuscript "Stress-induced DLK-vesicle assembly acts as local signaling platform that drives kinase activation and neurodegeneration" by Elena Tortosa et al. examines the localization and function of the MAP kinase dual leucine zipper kinase (DLK) in response to neuronal stress induced by neuronal growth factor removal. The authors examine phosphorylation of a number of kinases and find that DLK is the only one examined that increases in response to NGF removal. They also show that DLK membrane association increases in response to the stressor. Previous work has shown that DLK is lipid-modified by palmitate, which is necessary for association with vesicles and downstream signaling. The authors' data
largely agree with these previous findings. There are new findings, including that inhibition of internalization blocks DLK activity and that DLK needs a sphingomyelin-enriched compartment for activation. However, major flaws in the interpretation of these data need to be addressed before this manuscript is suitable for publication.

Major points:

1. The conclusion that DLK relies on endocytosis for activation is based, in large part, on use of the endocytosis inhibitor dynasore. However, dynasore is not a dynamin-specific inhibitor (Preta et al 2015). It has several major off-target mechanisms. It also interferes with cholesterol homeostasis, and cyclodextrin also disrupts normal cholesterol levels. Are the changes in DLK signaling due to alterations in cholesterol homeostasis? Other non-pharmacological approaches should be used to confirm this finding, as it is one of the main novel findings in this manuscript.

We agree with the reviewer that dynasore, although it is a widely used compound to inhibit dynamin and endocytosis, might have some off-target effects. To overcome this issue, we repeated some of the experiments with other compounds known to inhibit different endocytic pathways such as hypertonic sucrose media, chlorpromazine and dansylcadaverine as inhibitors of clathrin-dependent endocytosis, and methyl-b-cyclodextrin as an inhibitor of clathrin-independent and caveolin-dependent endocytosis pathway (Figure 5I-L, 6B, Appendix Figure S3K, L). In all cases we were able to block DLK vesicle localization and downstream signaling activation upon NGF withdrawal. In the revised version of the manuscript, these pharmacological treatments are supported by a genetic approach by overexpressing a mutant form of dynamin-1 (Dnm-1- K44A) that is defective in GTP hydrolysis and acts as a dominant negative form for dynamin (Van der Bliek et al, 1993). We observe how, under overexpression of this mutant form of dynamin, we are able to block the activation of caspase-3 and the process of axon degeneration induced upon NGF withdrawal (see new Figure 6C and F).

Cholesterol homeostasis and endocytosis are two interrelated pathways: cholesterol depletion has been shown to have diverse effects on the endocytic pathways and endocytosis is essential for cellular cholesterol transport. Therefore, it is difficult to rule out if the effect we observe with dynasore or cyclodextrin treatments in the DLK signaling pathway are due to alterations in cholesterol homeostasis or to the effect that this cholesterol change has on the endocytic pathway. However, the fact that other treatments that do not directly affect cholesterol homeostasis such as hypertonic sucrose media or dansylcadaverine, and the new genetic approach using a dominant negative form of dynamin-1 have similar effects on DLK signaling pathway suggests that the effects we observe are controlled by the endocytic pathway and not by cholesterol levels.

2. Another major finding was that DLK associates with a sphingomyelin-enriched compartment. The authors use immunoprecipitation with HA alone, WT DLK-HA, or non-palmitoylatable DLK-HA and then subsequent lipid analysis from the immunoprecipitated fraction. However, the authors show by immunostaining, blot, and IP that HA alone does not express in HeLa cells. In addition, non-palmitoylatable DLK has been shown to be cytoplasmic, rather than lipid-associated (Holland et al), consistent with the results in this manuscript (Figure 3E). Given these points, it is hard to understand how the authors are pulling down any vesicles at all in the HA alone or palmitoylation dead DLK conditions, let alone enough for lipid analysis. This raises serious doubts about the specificity of this assay.

We agree with the reviewer that the enrichment for DLK-associated lipids compared to HA-tag control is quite weak. We followed the protocol published for immunoprecipitation of lysosomes (Abu-Remaileh et al, 2017) in which no detergent is used in any step. This allows to keep intact intracellular organelles but, on the other hand, results in a high background noise. We think the signal observed in HA and DLK-CS is probably due to the unspecific binding of lipids to the beads or the DLK protein. In the revised manuscript, we have improved the protocol and greatly reduced the background signal (see new Figure 4E and F). Additionally, we think that HA tag alone is too small to be detected by regular methods such as Western blot or immunofluorescence. It is not retained in the Western blot membrane and probably will diffuse out of the cell after permeabilization for immunostainings.
3. It is not clear how the sphingomyelin synthase inhibitor D609 is working in these experiments. The authors conclude that sphingomyelin is necessary for DLK activation and downstream signaling. However, D609 seems to be also either significantly reducing (Fig 4G) or completely abolishing (Fig 4K) levels of DLK. This complicates interpretation of this experiment.

We thank the reviewer for pointing this out. D609 treatments do not reduce DLK protein levels as it is quantified in Figure 4I. Additionally, we have analyzed the remaining fraction we get from our fractionation assays that correspond to an insoluble fraction and have seen that DLK accumulates in this insoluble fraction under D609 treatments (see new Figure 4L).

4. Kymographs are useful for following movement of vesicles, but it would be more useful to see standard images of neurons under all of the conditions. One might expect DLK to become more diffuse and cytoplasmic when not active or palmitoylated, as Holland et al reported, but it is not possible to see that in kymograph format.

We agree with the reviewer and images of neurons have been added in the revised version of the manuscript. As the reviewer indicated, you can see how DLK become more diffused under treatments that affect its localization as it is the case for D609 or dynasore treatments.

5. Colocalization might be more accurately measured using an object-based colocalization, since much of the image is black when imaging vesicle-associated proteins.

The reviewer is correct – we have now performed object-based colocalization analysis and the results have been included in Figure 4A and B.

6. Incorrect or confusing statistics are being used. For example, Figure 5B is using one-way ANOVA but changing two independent variables. Please check statistical tests throughout.

We thank the reviewer for pointing this out. We have corrected this analysis in the revised version of the manuscript and repeated or explained better in the figure legends for the other figures.

Minor points:

1. Please remove the reference to trending in the section below. If the data are not significantly different, they are not different. "The increased levels of phosphatidylethanolamine and phosphatidylcholine in the DLK fraction is trending, however only sphingomyelin levels and species were significantly different (Figure 4E, F)."

We have removed the sentence in the revised version of the manuscript.

2. Figure 1E does not really add to the manuscript, since the authors are not focusing broadly on kinase palmitoylation, but specifically on DLK.

We have removed Figure 1E as requested.

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Thank you for submitting your revised manuscript. Your revision has now been seen by the referees and as you can see below, they appreciate the introduced changes and support publication here. Please include the suggested citation (referee #1).

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Referee #1:

The authors have addressed my concerns from the previous review. Since the initial review and perhaps since the resubmission, there is a new paper out that investigates DLK signaling and the role of palmitoylation in optic nerve injury. (PMID: 35349303). It would be timely for the authors to discuss how these separate study complement their findings in the discussion.

Referee #2:
The authors have addressed all points raised adequately, either through changes in data presentation, analysis or interpretation or through text changes. The Discussion section is now more closely associated with the experimental findings and relates them better to findings in the field.
No further changes are requested by this reviewer.

Referee #3:

This manuscript by Tortosa et al. examines the mechanisms regulating the activation of DLK during neuronal stress. The authors were responsive to previous critiques and the manuscript is much improved. The results are significant for the field and the data quality is excellent. I support publication of this manuscript in its current form.

2nd Authors' Response to Reviewers 21st Apr 2022

The authors have made all requested editorial changes.
Dear Casper,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a look at it and all looks good. I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study

Best Karin

Karin Dumstrei, PhD
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