

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

Elena Tortosa, Arundhati Sengupta Ghosh, Qingling Li, Weng Wong, Trent Hinkle, Wendy Sandoval, Christopher Rose, and Casper Hoogenraad

DOI: 10.15252/emboj.2021110155

Corresponding author(s): Casper Hoogenraad (c.hoogenraad@uu.nl) , Casper Hoogenraad (c.hoogenraad@uu.nl)

Review Timeline:

Submission Date:	9th Nov 21
Editorial Decision:	16th Dec 21
Revision Received:	15th Mar 22
Editorial Decision:	13th Apr 22
Revision Received:	21st Apr 22
Accepted:	22nd Apr 22

Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

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

Guide For Authors: <https://www.embopress.org/page/journal/14602075/authorguide>

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:

<https://emboj.msubmit.net/cgi-bin/main.plex>

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/ $\mu\text{m}^2 \cdot \text{min}$ 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 the reader draw 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. The

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.

Point-to-point rebuttal - RE: EMBOJ-2021-110155 "Stress-induced DLK assemblies act as signaling hubs to drive kinase activation and neurodegeneration"***** 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.

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/ $\mu\text{m} \cdot \text{min}$ 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.

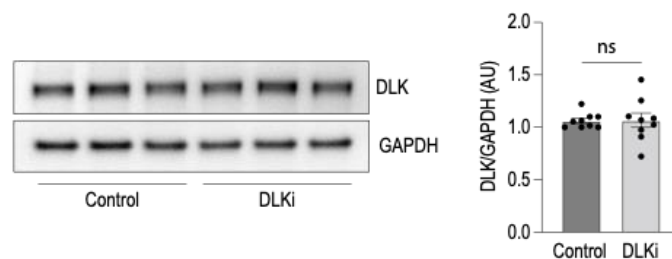


Figure 1. DLK inhibitor treatments do not affect DLK protein levels. Representative Western blots of DLK and GAPDH (left) and quantification of DLK protein levels (right) from 3DIV cultured embryonic DRG neurons treated with DLK inhibitor (DLKi) for 3h.

*** 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 the reader 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 *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, eg. 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 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?

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- β -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.

REFERENCES:

- Abu-Remaileh M, Wyant GA, Kim C, Laqtom NN, Abbasi M, Chan SH, Freinkman E & Sabatini DM (2017) Lysosomal metabolomics reveals V-ATPase- and mTOR-dependent regulation of amino acid efflux from lysosomes. *Science* (80-) 358
- Van der Bliek AM, Redelmeier TE, Damke H, Tisdale EJ, Meyerowitz EM & Schmid SL (1993) Mutations in human dynamin block an intermediate stage in coated vesicle formation. *J Cell Biol* 122
- Chen X, Rzhetskaya M, Kareva T, Bland R, During MJ, Tank AW, Kholodilov N & Burke RE (2008) Antiapoptotic and trophic effects of dominant-negative forms of dual leucine zipper kinase in

- dopamine neurons of the substantia nigra in vivo. *J Neurosci* 28
- Fernandes KA, Harder JM, John SW, Shrager P & Libby RT (2014) DLK-dependent signaling is important for somal but not axonal degeneration of retinal ganglion cells following axonal injury. *Neurobiol Dis* 69
- Hu Z, Deng N, Liu K & Zeng W (2019) DLK mediates the neuronal intrinsic immune response and regulates glial reaction and neuropathic pain. *Exp Neurol* 322
- Ma J, Goodwani S, Acton PJ, Buggia-Prevot V, Kesler SR, Jamal I, Mahant ID, Liu Z, Mseeh F, Roth BL, *et al* (2021) Inhibition of dual leucine zipper kinase prevents chemotherapy-induced peripheral neuropathy and cognitive impairments. *Pain* 162
- Miller BR, Press C, Daniels RW, Sasaki Y, Milbrandt J & Diantonio A (2009) A dual leucine kinase-dependent axon self-destruction program promotes Wallerian degeneration. *Nat Neurosci* 12: 387–389
- Patel S, Cohen F, Dean BJ, De La Torre K, Deshmukh G, Estrada AA, Ghosh AS, Gibbons P, Gustafson A, Huestis MP, *et al* (2015) Discovery of dual leucine zipper kinase (DLK, MAP3K12) inhibitors with activity in neurodegeneration models. *J Med Chem* 58
- Le Pichon CE, Meilandt WJ, Dominguez S, Solanoy H, Lin H, Ngu H, Gogineni A, Ghosh AS, Jiang Z, Lee SH, *et al* (2017) Loss of dual leucine zipper kinase signaling is protective in animal models of neurodegenerative disease. *Sci Transl Med* 9
- Pozniak CD, Ghosh AS, Gogineni A, Hanson JE, Lee SH, Larson JL, Solanoy H, Bustos D, Li H, Ngu H, *et al* (2013) Dual leucine zipper kinase is required for excitotoxicity-induced neuronal degeneration. *J Exp Med* 210: 2553–2567
- Watkins TA, Wang B, Huntwork-Rodriguez S, Yang J, Jiang Z, Eastham-Anderson J, Modrusan Z, Kaminker JS, Tessier-Lavigne M & Lewcock JW (2013) DLK initiates a transcriptional program that couples apoptotic and regenerative responses to axonal injury. *Proc Natl Acad Sci U S A* 110: 4039–4044
- Welsbie DS, Mitchell KL, Jaskula-Ranga V, Sluch VM, Yang Z, Kim J, Buehler E, Patel A, Martin SE, Zhang PW, *et al* (2017) Enhanced Functional Genomic Screening Identifies Novel Mediators of Dual Leucine Zipper Kinase-Dependent Injury Signaling in Neurons. *Neuron* 94: 1142-1154.e6
- Welsbie DS, Yang Z, Ge Y, Mitchell KL, Zhou X, Martin SE, Berlinicke CA, Hackler L, Fuller J, Fu J, *et al* (2013) Functional genomic screening identifies dual leucine zipper kinase as a key mediator of retinal ganglion cell death. *Proc Natl Acad Sci U S A* 110
- Welsbie DS, Ziogas NK, Xu L, Kim BJ, Ge Y, Patel AK, Ryu J, Lehar M, Alexandris AS, Stewart N, *et al* (2019) Targeted disruption of dual leucine zipper kinase and leucine zipper kinase promotes neuronal survival in a model of diffuse traumatic brain injury. *Mol Neurodegener* 14
- Wlaschin JJ, Gluski JM, Nguyen E, Silberberg H, Thompson JH, Chesler AT & Le Pichon CE (2018) Dual leucine zipper Kinase is required for mechanical allodynia and microgliosis after nerve injury. *Elife* 7: 1–19

Dear Casper,

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).

When you submit the revised manuscript will you also take care of the following points:

- You can only have 3-5 keywords
- Please make the mass spec data deposit open and remove referee password
- Please re-label COI as "Disclosure statement and competing interests"
- We are using the CRediT author contribution format, which allows for systematic, machine-readable author contributions and thus more effective research assessment, while also allowing addition of any relevant further information for each author. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. See also guide to authors "Author Contributions"
- I don't think any funding information has been added - please double check.
- Appendix Table S1 should be named Dataset EV1, and the name and legend (as separate tab) added to the file
- Please include page numbers in the appendix.
- The source data needs to be merged as one file per figure, or where there are multiple file types to one folder per figure.
- The Reagents and Tools table should be uploaded separately using the file type 'Reagents Table'.
- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Please take a look at the word file and the comments regarding the figure legends and respond to the issues.

That should be all! Let me know if you have any questions

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Guide For Authors: <https://www.embopress.org/page/journal/14602075/authorguide>

Use the link below to submit your revision:

<https://emboj.msubmit.net/cgi-bin/main.plex>

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
Senior Editor
The EMBO Journal

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here:
<https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess>

Your manuscript will be processed for publication in the journal by EMBO Press. Manuscripts in the PDF and electronic editions of The EMBO Journal will be copy edited, and you will be provided with page proofs prior to publication. Please note that supplementary information is not included in the proofs.

Please note that you will be contacted by Wiley Author Services to complete licensing and payment information. The required 'Page Charges Authorization Form' is available here: https://www.embopress.org/pb-assets/embo-site/tej_apc.pdf

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

** Click here to be directed to your login page: <https://emboj.msubmit.net>

EMBO Press Author Checklist

Corresponding Author Name: Casper Hoogenraad
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2021-110155R

USEFUL LINKS FOR COMPLETING THIS FORM

[The EMBO Journal - Author Guidelines](#)
[EMBO Reports - Author Guidelines](#)
[Molecular Systems Biology - Author Guidelines](#)
[EMBO Molecular Medicine - Author Guidelines](#)

Reporting Checklist for Life Science Articles (updated January 2022)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: [10.31222/osf.io/9sm4x](#)). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- ☒ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ☒ ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- ☒ plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ☒ if $n < 5$, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- ☒ Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- ☒ a specification of the experimental system investigated (eg cell line, species name).
- ☒ the assay(s) and method(s) used to carry out the reported observations and measurements.
- ☒ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ☒ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- ☒ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ☒ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ☒ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ☒ definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of "center values" as median or average;
 - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	"Contact for reagent and resource sharing" in Materials and Methods section
Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For antibodies provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and/or clone number - Non-commercial: RRID or citation	Yes	In Reagents and Tools Table
DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	In Reagents and Tools Table
Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository OR supplier name, catalog number, clone number, and/OR RRID.	Yes	In Reagents and Tools Table
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	"Experimental models" In Materials and Methods section
Report if the cell lines were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	In Materials and Methods section
Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository OR supplier name, catalog number, clone number, OR RRID.	Yes	In Materials and Methods section
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail housing and husbandry conditions.	Yes	In Materials and Methods section
Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	
Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	
Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been pre-registered , provide DOI in the manuscript. For clinical trials, provide the trial registration number OR cite DOI.	Not Applicable	
Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	
Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if external detailed step-by-step protocols are available.	Not Applicable	
Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)

Include a statement about sample size estimate even if no statistical methods were used.	Yes	In Figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, have they been described?	Not Applicable	
Include a statement about blinding even if no blinding was done.	Yes	In Materials and Methods
Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Yes	In Materials and Methods
If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.		
For every figure, are statistical tests justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	In Figure legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was replicated in laboratory.	Yes	In Figure legends
In the figure legends: define whether data describe technical or biological replicates .	Yes	In Figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving human participants : State details of authority granting ethics approval (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving human participants : Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving human participants : For publication of patient photos , include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental animals : State details of authority granting ethics approval (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	In Materials and Methods section, Experimental Models, Animals
Studies involving specimen and field samples : State if relevant permits obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of select agents and toxins (CDC): https://www.selectagents.gov/sa/list.html .	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the authority granting approval and reference number for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., ICMJE, MIBBI, ARRIVE, PRISMA) have been followed or provided.	Not Applicable	
For tumor marker prognostic studies , we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For phase II and III randomized controlled trials , please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have primary datasets been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability Section
Were human clinical and genomic datasets deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are computational models that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective data citations in the reference list .	Not Applicable	