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Oligomerisation-driven MLKL ubiquitylation antagonises necroptosis

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1st Editorial Decision 29th Nov 2019

Dear John,

Thank you for submitting your manuscript entitled "Oligomerization-driven MLKL ubiquitylation antagonizes necroptosis" [EMBOJ-2019-103718] to The EMBO Journal. Please accept my sincere apologies for the unusual length of the reviewing, due to the delayed delivery of one report and detailed discussions within the team. The manuscript has now been reviewed by two referees, whose comments are provided below. In light of these reports, I am afraid that the study is not a sufficiently strong candidate for publication here.

As you can see, the referees write that the role of MLKL ubiquitylation in suppressing necroptosis and the molecular mechanism(s) thereof would need to be further investigated. In particular, they find that it is important to discriminate if phosphorylation and ubiquitylation of MLKL occur at the plasma membrane or in RIPK3-insoluble aggregates. In addition, the reviewers are concerned about the interpretation of the data on MLKL ubiquitylation in presence of wild-type and inactive USP21. Given these opinions from trusted experts in the field, and the large number of additional experiments requested to strengthen the findings, I am afraid that we cannot offer to invite a revised version of your manuscript at this stage.

However, considering the relevance of your data, we remain interested in the study and would be willing to reconsider it as a new submission at a later time, should the referees' concerns and suggestions be duly addressed. Please note that the novelty of your study will be taken into consideration at the time of resubmission.

I thank you for the opportunity to consider this manuscript. While I am sorry that I could not communicate more positive news, I hope that the referees' comments will be helpful for you to improve the manuscript.

Best regards,

Elisabetta

Elisabetta Argenzio, PhD
Editor
The EMBO Journal

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Referee #1:

Liu et al investigate how ubiquitylation of MLKL affects necroptosis. Fig. 1 establishes ubiquitylation of MLKL in mouse or human cells in response to a necroptosis stimulus. Fig. 2 provides evidence for mono-ubiquitylation of MLKL at multiple sites taking place in a crude fraction that includes membranes and amyloid-like aggregates. One major criticism is that the abstract ignores the latter and focuses on membranes as the site of MLKL ubiquitylation (see major point #1 below).

Fig. 3 indicates that ubiquitylation of MLKL coincides with oligomerization but RIPK3 is not strictly required. Fig. 4 shows that tagging MLKL at the N-terminus with a Flag epitope, which inactivates its ability to kill cells, enhances its turnover via the proteasome or lysosome. The relevance of this observation to wild-type, untagged MLKL is less clear. Fig. 5 shows that mutation of the ubiquitylation sites in Flag-MLKL did not prevent ubiquitylation of untagged MLKL. Thus, the sites were either not relevant, or as is often the case, there is promiscuity in the ubiquitylation sites that can be used.

Next the authors fused MLKL to the catalytic domain of USP21 (wild-type or inactive) as a strategy to eliminate ubiquitylation on MLKL and assess the functional consequence. Unfortunately, the data are not so black and white. Fig. 5D fails to convince me that either fusion is ubiquitylated (major point #2 below), which makes interpretation of the survival curves in Fig. 5E problematic. Therefore, the case for ubiquitylation of MLKL suppressing necroptosis still needs some work.

MAJOR POINTS:
1) Both ubiquitylation and phosphorylation of MLKL occurs in the crude membrane fraction (Fig. 3C). We know that active RIPK3 forms insoluble aggregates. Thus, both phosphorylation and ubiquitylation of MLKL may occur in these macromolecular structures rather than at membranes. The authors allude to this on line 14 (3rd page of results), but they call out ubiquitylation of MLKL on biological membranes in their abstract. Resolution of aggregates vs. membranes seems warranted.
2) Fig. 5D is used to claim that MLKL fused to the wild-type USP21 catalytic domain is not ubiquitylated in response to TSI, whereas the fusion using the inactive version of USP21 is ubiquitylated. I just don't see this difference. A ubiquitin ladder is not obvious on either protein and the upward smears seem more a function of protein abundance. Would MS better illuminate the claimed differences?

MINOR POINTS:
1) Fig. 1B, why is p-MLKL detected in TNFR1 KO cells after TSI treatment? Does SI activate MLKL independent of TNFR1?
2) Line 24 (1st page of results) suggests ubiquitylation of RIPK1 and RIPK3 in cells given TSI (Fig. 1B). While this would be consistent with what has been reported by others, this statement isn't well supported by the data in Fig. 1B. i.e. there isn't clear laddering for either RIPK1 or RIPK3 in the UBA pull-downs. There is a slight upshift for RIPK3, but can the authors exclude that this isn't just due to phosphorylation? The presence of RIPK1 and RIPK3 in the pull-downs could merely indicate their association with ubiquitylated proteins.

Referee #2:

The authors report that MLKL is ubiquitylated specifically during necroptosis induction, dependent on its oligomerization and association with crude membrane fractions. They propose that this modification is characterized by mono-ubiquitylation at multiple lysines, which can only be removed with USP21. Inactive mutants of MLKL that are ubiquitylated are degraded via the proteasome and lysosome. Fusion of MLKL to USP21 generated a non-ubiquitylated MLKL that was cytotoxic than MLKL fused to an inactive form of USP21. Based on this the authors propose that MLKL ubiquitylation at the membrane upon activation contributes to decrease the levels of active MLKL and therefore limits its necroptosis-inducing activity.

The study is clearly written and although the regulation of protein levels by ubiquitylation is hardly novel, the authors detect this modification only upon MLKL oligomerization and membrane translocation. This supports a role of ubiquitylation in the control of the levels of cytotoxic MLKL that would be more interesting. However, it turns out that ubiquitylation of MLKL is not required for necroptosis and just provides a regulatory level in the pathway which affects the kinetics of cell death. To make a more compelling story out of this study, the authors should provide significant additional insight into the molecular mechanisms that mediate MLKL ubiquitylation and test their role in necroptosis regulation.

Major concerns:

1. Additional mechanistic understanding is required: What ubiquitylates MLKL? Does depletion of this E3 ligase affect necroptosis? What is the molecular mechanism that allows specific modification of MLKL oligomers at the membrane? Would cytosolic MLKL oligomers be ubiquitylated? In which cellular membranes is ubiquitylated MLKL found? Is MLKL mono-ubiquitylation associated to endosome-trafficking?

2. The authors show that proteasome and lysosome inhibition leads to accumulation of ubiquitylated inactive mutant MLKL, and propose that degradation of ubiquitylated MLKL regulates necroptosis. Accordingly, does proteasome and/or lysosome inhibition during wt MLKL activation lead to an accumulation of wtMLKL and to an increase in the kinetics of cell death? The authors should find some way to determine whether ubiquitylation of wt active MLKL also contributes to its proteasomal degradation.

3. The authors should demonstrate that the assays with DUBs work, but showing deubiquitylation with a control protein.

4. The authors show that constitutively active MLKL becomes ubiquitylated. They could complement this experiments with dimerizable MLKL.

5. Why are USP21 fusion slower to kill than wt MLKL? Is there an effect of the fusion on the activity? What is the origin of the stimulus-independent activity of MLKL-USP21? Increased expression levels? Decreased proteasome/lysosome degradation? Authors concluded that this intrinsic activity of MLKL-USP21 could be the result of its stimulus-independent phosphorylation in MLKL KO cells. However, similar effect is observed in RIPK3/MLKL DKO cells. Which is the explanation here?

6. The authors mostly provide detailed information about the ubiquitylation mechanism of mouse MLKL; however, information about human MLKL is missing. As they mentioned, they and others have demonstrated that regulation of human and mouse MLKL is quite different and they show in the manuscript that human MLKL can also be ubiquitylated during necroptosis. Is human MLKL also ubiquitylated at multiple sites? Which is the ubiquitin architecture of human MLKL? Does ubiquitination of human MLKL require translocation to membranes and oligomerization? WB equivalents to those shown for mouse MLKL (figure 2C) should be included. The authors show that NSA affects human MLKL ubiquitylation. However, in their hands this inhibitor does not affect MLKL oligomerization. Which would be the effect of mutations that affect oligomerization and membrane translocation of human MLKL (equivalent to R105 and D106 in mouse)? Is human MLKL ubiquitylation also independent on RIPK3 or extrinsic necroptotic stimuli? Intrinsically active mutants of human MLKL (e.g. phosphomimetics) should also be tested. Is human MLKL

ubiquitylation dispensable for cell death?

Minor issues:

6. The western blot in figure 3A should be improved.

EMBOJ-2019-103718– Response to Reviewers

Corresponding authors: Hildebrand, JM and Silke J. The Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, 3052, Australia

We thank the reviewers for their thoughtful, constructive and detailed feedback on our manuscript. In response we have generated **ten** new panels of data that are highlighted with red boxes in the figures. We have also made appropriate changes in the main text and these are likewise indicated in colour for ease of reference. In our detailed response below, the reviewers' remarks are presented *verbatim* in black and our response to reviewers' comments are presented in *blue italics*.

Referee #1.

Liu et al investigate how ubiquitylation of MLKL affects necroptosis. Fig. 1 establishes ubiquitylation of MLKL in mouse or human cells in response to a necroptosis stimulus. Fig. 2 provides evidence for mono-ubiquitylation of MLKL at multiple sites taking place in a crude fraction that includes membranes and amyloid-like aggregates. One major criticism is that the abstract ignores the latter and focuses on membranes as the site of MLKL ubiquitylation (see major point #1 below).

We agree that this is an important point to address in the abstract and have replaced "crude membrane fraction" with the line "digitonin insoluble cell fraction comprising plasma/organellar membranes and protein aggregates" on ***p2, lines 8-9.***

Fig. 3 indicates that ubiquitylation of MLKL coincides with oligomerization but RIPK3 is not strictly required. Fig. 4 shows that tagging MLKL at the N-terminus with a Flag epitope, which inactivates its ability to kill cells, enhances its turnover via the proteasome or lysosome. The relevance of this observation to wild-type, untagged MLKL is less clear.

We agree that Flag-tagged, exogenously expressed MLKL is a step removed from the 'real life' situation. We have now more clearly articulated our rationale for using this construct, which was two-fold; N-terminal FLAG tagged MLKL behaves like WT untagged MLKL up until the point of membrane lysis (that is, it is phosphorylated by RIPK3 and forms high molecular weight oligomers that are localised to 0.025% insoluble phases of the cell) but precludes cell death. This feature of N-FLAG MLKL allows accumulation of large amounts of ubiquitylated MLKL in situ in living cells, and has been crucial to the high quality affinity purification (via the FLAG tag) of sufficient levels of ubiquitylated MLKL for Mass Spec analyses. Given our observations of MLKL ubiquitylation on N-Flag MLKL support and complement our observations derived from endogenous MLKL, other MLKL point mutants and MLKL-USP21 fusion, we believe that N-FLAG MLKL is a useful and relevant tool for the study of MLKL at all steps prior to membrane destruction. To clearly address this point for readers, we have added the following at the point that N-FLAG-MLKL is first introduced;

*"and N-terminal FLAG-tagged MLKL (N-FLAG MLKL) are able to form higher order oligomers in crude membrane fractions following necroptotic stimulation, yet are nevertheless unable to induce necroptosis (Hildebrand et al., 2014, Tanzer et al., 2016) (****Fig. 5, B, Supp. Fig. 5A, B****). ****P9, line 11-14****."*

Fig. 5 shows that mutation of the ubiquitylation sites in Flag-MLKL did not prevent ubiquitylation of untagged MLKL. Thus, the sites were either not relevant, or as is often the case, there is promiscuity in the ubiquitylation sites that can be used.

Next the authors fused MLKL to the catalytic domain of USP21 (wild-type or inactive) as a strategy to eliminate ubiquitylation on MLKL and assess the functional consequence. Unfortunately, the data are not so black and white. Fig. 5D fails to convince me that either fusion is ubiquitylated (major point #2 below), which makes interpretation of the survival curves in Fig. 5E problematic. Therefore, the case for ubiquitylation of MLKL suppressing necroptosis still needs some work.

Please see response to Major point #2 below.

MAJOR POINTS:

1) Both ubiquitylation and phosphorylation of MLKL occurs in the crude membrane fraction (Fig. 3C). We know that active RIPK3 forms insoluble aggregates. Thus, both phosphorylation and ubiquitylation of MLKL may occur in these macromolecular structures rather than at membranes. The authors allude to this on line 14 (3rd page of results), but they call out ubiquitylation of MLKL on biological membranes in their abstract. Resolution of aggregates vs. membranes seems warranted.

We agree that this is an important point. We have looked for physical fractionation methods that can accurately separate cytosolic protein aggregates from biological membranes but couldn't find any suitable for this purpose. For example, owing to the ubiquitous nature of ubiquitin throughout the cell, we expect that signal overlap or proximity amplified detection methods (e.g. proximity ligation assays) would be very difficult to optimize for the detection of direct MLKL ubiquitylation by imaging.

*However, we have added a figure panel (**Fig. 3B**) showing that MLKL-ubiquitylation can be reduced by USP21 which is fused with the biological membrane targeting -CaaX motif. We discuss this result in more detail in the manuscript but it implies that a proportion of ubiquitylated MLKL is present at the plasma membrane, though we cannot conclude if it is generated there. We also present new data in **Supp. Fig. 3** to show that this USP21-CaaX fusion protein did not alter the original signalling pathways for either apoptosis or necroptosis.*

2) Fig. 5D is used to claim that MLKL fused to the wild-type USP21 catalytic domain is not ubiquitylated in response to TSI, whereas the fusion using the inactive version of USP21 is ubiquitylated. I just don't see this difference. A ubiquitin ladder is not obvious on either protein and the upward smears seem more a function of protein abundance. Would MS better illuminate the claimed differences?

*We have optimised protein separation conditions such that the ubiquitin ladders on mouse MLKL-USP21 fusions used in **Fig. 5D** (original submission) are much more clearly resolved (see new **Fig. 7B**). We have also gone further to show that the same distinctive ubiquitin ladder can be seen with human MLKL-USP21 fusions in the human HT29 cell line (see new **Fig. 7D**). Furthermore, in our new **Fig. 7E** we also demonstrate that these human MLKL-USP21 fusions not only have the same propensity for accelerated death following TSI, relative to human MLKL-catalytically-dead USP21 fusions or MLKL alone, but also auto-activate in the absence of TSI. This is an extremely interesting new finding because we and others have shown that human MLKL, unlike mouse MLKL, is very resistant to attempts to make it auto-active. Thus*

expression of the N-terminal domain (NTD) of human MLKL alone is not toxic to cells (without enforcing oligomerization) while murine NTD expression is. Similarly, a phospho-mimetic mutant of murine MLKL is auto-active (Murphy et al, 2013), while phospho-mimetic mutants of human MLKL are not only not auto-active, they are also loss of function (Petrie et al, 2018; Tanzer et al., 2016). This suggests that ubiquitylation is a particularly important means of regulating human MLKL's killing activity.

MINOR POINTS:

1) Fig. 1B, why is p-MLKL detected in TNFR1 KO cells after TSI treatment?

*We too were intrigued by the presence of low levels of p-MLKL post TSI treatment of Tnfr1$^{-/-}$ cells in **Fig. 1B**. It is possible that Smac-mimetic and IDN-6556 alone are promoting the formation of a Ripoptosome, at the time we did not follow up on this because of the lack of MLKL-ubiquitylation. We have noted this observation on **p5, lines 19-20**, but believe that further speculation about this observation may detract from the main message of our manuscript.*

Does SI activate MLKL independent of TNFR1?

*We commonly observe some MLKL activation (indicated by presence of phospho-MLKL) following stimulation with SI alone in cells of hematopoietic origin (**Fig. 1C**). In MDFs phospho-MLKL is less pronounced, or as in **Fig. 1A**, absent. However one can imagine that if the phospho-MLKL blot was exposed to the same level as in **Fig. 1B,** it might be observable. We attribute this to the production of TNF by target cells in response to Smac-mimetic induced activation of the alternative NF-kappa B signalling pathway as described by our team and others in 2007 (Vince et al, 2007). We have clarified this point for readers on **P5, line 29-31***

2) Line 24 (1st page of results) suggests ubiquitylation of RIPK1 and RIPK3 in cells given TSI (Fig. 1B). While this would be consistent with what has been reported by others, this statement isn't well supported by the data in Fig. 1B. i.e. there isn't clear laddering for either RIPK1 or RIPK3 in the UBA pull-downs. There is a slight upshift for RIPK3, but can the authors exclude that this isn't just due to phosphorylation? The presence of RIPK1 and RIPK3 in the pull-downs could merely indicate their association with ubiquitylated proteins.

*We agree that the data do not unambiguously support our statement and we have therefore removed the line 'Interestingly, RIPK1 and RIPK3 also undergo ubiquitylation following TSI stimulation, which was reduced in Traf2$^{-/-}$ MDFs (**Fig. 1B**).*

Referee #2.

The authors report that MLKL is ubiquitylated specifically during necroptosis induction, dependent on its oligomerization and association with crude membrane fractions. They propose that this modification is characterized by mono-ubiquitylation at multiple lysines, which can only be removed with USP21. Inactive mutants of MLKL that are ubiquitylated are degraded via the proteasome and lysosome. Fusion of MLKL to USP21 generated a non-ubiquitylated MLKL that was cytotoxic than MLKL fused to an inactive form of USP21. Based on this the authors propose that MLKL ubiquitylation at the membrane upon activation contributes to decrease the levels of active MLKL and therefore limits its necroptosis-inducing activity.

The study is clearly written and although the regulation of protein levels by ubiquitylation is hardly novel, the authors detect this modification only upon MLKL oligomerization and membrane translocation. This supports a role of ubiquitylation in the control of the levels of cytotoxic MLKL that would be more interesting. However, it turns out that ubiquitylation of MLKL is not required for necroptosis and just provides a regulatory level in the pathway which affects the kinetics of cell death. To make a more compelling story out of this study, the authors should provide significant additional insight into the molecular mechanisms that mediate MLKL ubiquitylation and test their role in necroptosis regulation.

Major concerns:

1. Additional mechanistic understanding is required: What ubiquitylates MLKL? Does depletion of this E3 ligase affect necroptosis? What is the molecular mechanism that allows specific modification of MLKL oligomers at the membrane? Would cytosolic MLKL oligomers be ubiquitylated? In which cellular membranes is ubiquitylated MLKL found? Is MLKL mono-ubiquitylation associated to endosome-trafficking?

These are all excellent questions and we have made exhaustive attempts using both mass spectrometry and fluorescence microscopy over several years to answer them. Despite detecting potential E3 ligase candidates following MLKL affinity pull down and Mass Spec analysis, CRISPR-Cas9 induced single gene knock-out cell lines retained full capacity for MLKL ubiquitylation (data not shown). A couple of potential explanations are that the E3 ligase interaction might be insufficiently stable to detect via IP/Mass Spectrometry or that there is a level of redundancy that prevents us from singling out a single specific E3 ligase for MLKL. Whatever the reason it is not from want of trying that we have failed to identify an E3 responsible for this ubiquitylation.

As mentioned above in our response to reviewer 1, we have met several road blocks when it comes to distinguishing the precise subcellular location of ubiquitylated MLKL – the most important of which is the specific detection of ubiquitylated MLKL by immunohistochemistry as opposed to total MLKL (which includes non-ubiquitylated forms). Certainly several groups have demonstrated the trafficking of p-MLKL into endosomes for subsequent degradation by the lysosome (Yoon et al, 2017; Zargarian et al, 2017). However, we have not been able to isolate endosomes of sufficient quantity and purity to perform UBA-pulldowns for the subsequent detection of ubiquitylated MLKL. Based on our experience, we predict that on leaving the plasma membrane, endosome-located ubiquitylated MLKL species are very short lived in the cell.

Nevertheless our new data, showing that MLKL can be deubiquitylated by a plasma membrane localised DUB ***(Fig. 3B****), and that both mouse and human MLKL-USP21 fusions are 'auto-active'-, go some way to showing, as the reviewer states, "that it is the cytotoxic form of MLKL" that is being regulated by ubiquitylation. Together with our recent work showing that MLKL takes some time to accumulate at the plasma membrane (Samson et al, 2020) this suggests that this is both a feasible and physiological mechanism to regulate MLKL induced killing.*

2. The authors show that proteasome and lysosome inhibition leads to accumulation of ubiquitylated inactive mutant MLKL, and propose that degradation of ubiquitylated MLKL regulates necroptosis. Accordingly, does proteasome and/or lysosome inhibition during wt MLKL activation lead to an accumulation of wt MLKL and to an increase in the kinetics of cell

death? The authors should find some way to determine whether ubiquitylation of wt active MLKL also contributes to its proteasomal degradation.

It is difficult to draw a conclusion from experiments looking the effects of proteasome inhibitors on cell death because inhibiting the proteasome creates a cellular stress that sensitises cells to cell death stimuli and to TNF induced cell death in particular. An additional complication is that our necroptotic cell death stimulus utilises Smac-mimetics which work by promoting proteasomal degradation of cIAPs, and it is also not clear how proteasome inhibition might affect this stimulus.

3. The authors should demonstrate that the assays with DUBs work, but showing deubiquitylation with a control protein.

We would gladly have performed this experiment if we hadn't suffered from severe limitations on the lab work we can perform due to Victoria's strict lockdown. However our source of these enzymes is the Komander lab and they perform stringent quality control on each preparation of DUBs used at the time of production and they are stored in accordance with recommendations (Hospenthal et al, 2015). We have provided this information as follows;

*"Each DUB specifically recognizes and removes a known subset of poly-ubiquitin chain type with validated activity (**Fig. 2A**) (Stafford et al, 2018)." **P6, line 25-27***

4. The authors show that constitutively active MLKL becomes ubiquitylated. They could complement this experiments with dimerizable MLKL.

We agree that dimerizable MLKL would complement our experiments, however we have used two separate constitutively activated murine MLKL mutants (Q343A and the phospho-mimetic S345D) and believe these are sufficient to demonstrate that forms of MLKL that are activated without the need for RIPK3 become ubiquitylated, suggesting that ubiquitylation is a consequence of activation. We have also recently shown that another auto-activated form of mouse MLKL, $MLKL^{D139V}$ – is ubiquitylated and rapidly turned over in a proteasome dependent manner (Hildebrand et al, 2020).

5. Why are USP21 fusion slower to kill than wt MLKL? Is there an effect of the fusion on the activity?

*As the reviewer notes there is a moderate delay in TSI induced cell death in $Mlkl^{-/-}$ MDFs reconstituted with either mouse MLKL-USP21 or MLKL-$USP21^{CR}$. As might be expected, this effect is slightly more pronounced in the catalytically inactive $USP21^{CR}$ fusion (Dox + TSI, compare white>green>blue rows in **Fig. 7C**) which is not toxic in itself (Dox compare blue with green rows). We hypothesise that this is due to some alteration in the kinetics of MLKL oligomerization conferred by the large C terminal 50kDa fusion, but we were not able to resolve oligomers of these fusions by Blue Native PAGE to test this hypothesis. We did not attempt to fuse the USP21 to the N-terminal of MLKL because this, in our experience, precludes MLKL's killing activity. We included a GS linker to try and minimise stereo physical effects of the C-terminal fusion, so there are not any obvious adjustments/improvements that we can make to this series of constructs. With our new human MLKL constructs (**Fig. 7D, E P11, lines 23 onwards**) we see that the fusion to the catalytically inactive $USP21^{CR}$ mutant does not delay the onset of TSI induced cell death relative to unfused MLKL.*

What is the origin of the stimulus-independent activity of MLKL-USP21? Increased expression levels? Decreased proteasome/lysosome degradation? Authors concluded that this intrinsic activity of MLKL-USP21 could be the result of its stimulus-independent phosphorylation in MLKL KO cells. However, similar effect is observed in RIPK3/MLKL DKO cells. Which is the explanation here?

*This is an excellent point. We suggest that the best comparisons are between the MLKL- WT and mutant USP21 fusions, and when we examine expression levels of these two fusions they are almost identical (see **Fig. 7B**, anti-MLKL blot of whole cell lysate 'input'). Since MLKL levels are the same in both these fusions it is unlikely that expression levels are the reason for the stimulus independent activity. On the other hand, MLKL-USP21 is already phosphorylated even in the absence of TSI treatment while the MLKL-USP21^{CR} is not. Following TSI treatment, there is an increase in the pMLKL/MLKL ratio and rapid loss of actin which is consistent with the cell death observed in **Fig. 7C**. One interpretation of these results is that in living cells MLKL is phosphorylated by RIPK3 at constitutively low levels and this MLKL is cleared by ubiquitylation and proteasomal degradation, thus preventing MLKL from attaining levels required to kill cells. However, as the reviewer astutely observes, the MLKL-USP21 fusion kills even in Ripk3 knock-out cells. To our mind this indicates two, potentially connected, possibilities: either there are other kinases that can activate MLKL causing a conformational change resulting in activation, translocation and ubiquitylation, or that ubiquitylation is itself a mechanism to prevent a conformational change leading to auto-activation and phosphorylation.*

*To these original speculations we now have data from the human MLKL-USP21 fusions, both of which are expressed to similar levels (**Fig. 7D**). As we pointed out before, the phospho-mimetic human MLKL is completely inactive (Petrie et al., 2018; Tanzer et al., 2016), and the USP21 fusion is one of the only modifications that we are aware of, that renders human MLKL auto-activating (**Fig. 7E**). In this case however human MLKL-USP21 fusion is not phosphorylated until TSI is added to cells **(Fig. 7D**), even though it is auto-activating. Over a prolonged time course with a high dosage of doxycycline, there is a suggestion of low level MLKL phosphorylation in the new panel **Supp. Fig 7E** however it is also possible that this represents some slight cross activity to the high levels of non-phosphorylated MLKL. Given that mouse MLKL-USP21 can kill in Ripk3$^{-/-}$ cells and human MLKL-USP21 can kill without extensive phosphorylation we believe that the most parsimonious explanation of this data is that MLKL phosphorylation is an epiphenomenon that is not required for MLKL killing.*

*We propose that under normal cellular conditions, this low level of MLKL activation is efficiently cleared at biological membranes by ubiquitylation and lysosomal degradation. As further support of this scenario, we have included an additional data panel (**Supp. Figure. 7C**), demonstrating that low levels of phosphorylation at Ser345 can also be observed for N-FLAG WT MLKL when expressed in Ripk3$^{-/-}$Mlkl$^{-/-}$ MDFs. The fact that this RIPK3-independent phosphorylation is only detectable in the presence of the proteasome inhibitor PS341 indicates that ubiquitylation is an important mechanism for the tonic turn-over of these particular RIPK3-independent pMLKL species. This new data is now described on **P11 lines 17-19**.*

6. The authors mostly provide detailed information about the ubiquitylation mechanism of mouse MLKL; however, information about human MLKL is missing. As they mentioned, they and others have demonstrated that regulation of human and mouse MLKL is quite different

and they show in the manuscript that human MLKL can also be ubiquitylated during necroptosis. Is human MLKL also ubiquitylated at multiple sites? Which is the ubiquitin architecture of human MLKL? Does ubiquitination of human MLKL require translocation to membranes and oligomerization? WB equivalents to those shown for mouse MLKL (figure 2C) should be included. The authors show that NSA affects human MLKL ubiquitylation. However, in their hands this inhibitor does not affect MLKL oligomerization. Which would be the effect of mutations that affect oligomerization and membrane translocation of human MLKL (equivalent to R105 and D106 in mouse)? Is human MLKL ubiquitylation also independent on RIPK3 or extrinsic necroptotic stimuli? Intrinsically active mutants of human MLKL (e.g. phosphomimetics) should also be tested. Is human MLKL ubiquitylation dispensable for cell death?

*We agree with the reviewer and are grateful for the suggestion because it prompted a very interesting finding. To address this point we generated, as described above, human MLKL-USP21 fusion constructs. New data generated with these constructs shows that, like mouse MLKL, fusion to USP21 prevents human MLKL ubiquitylation following TSI stimulation (**Fig. 7D, E**). As expected, fusion to USP21 doesn't appear to affect phosphorylation of MLKL, but as in the mouse fusion, the catalytically inactive USP21 mutation marginally decreases the killing activity of MLKL following TSI stimulation when compared with the untagged MLKL. On the other hand, fusion with the catalytically active USP21 seems to enhance MLKL's cytotoxicity in response to TSI, which is most evident with 20 ng/ml doxycycline indication and because NSA is less able to inhibit cell death caused by the fusion MLKL (**Fig. 7E**).*

This probably reflects to some extent the fact that the hMLKL-USP21 fusion is auto-activating. Despite exhaustive attempts in multiple different human cells lines (HEK293T, HT29, U937, THP1), in our hands, phospho-mimetic mutants of human MLKL, when inducibly expressed in stably transfected cells do not show the stimulus independent, constitutive activity. Furthermore phospho-mimetic mutants of hMLKL are unable to be activated by TSI (thus in addition to showing that mMLKL and hMLKL appear to be regulated similarly we believe that this is an important finding for the field). Naturally we would have liked to examine the ubiquitin linkage type, amino acid sites of ubiquitylation and subcellular location of human MLKL, and look forward to pursuing this as a future direction when restrictions are eased.

Minor issues:

6. The western blot in figure 3A should be improved.

*We thank the reviewer for this suggestion and have now revised the text to underscore the key point of the western blot in **Fig. 3A (now Fig. 4A),** which is to verify the oligomerization or 'complex II' status of MLKL in the samples used for the UBA pull downs presented in **Fig. 3B (now Fig. 4B)**. The much reduced (undetectable) capacity for the MLKL R105AD106A mutant to form high molecular weight oligomers (complex II) - which is clearly shown here when comparing 'M' lanes following 'TSQ' stimulation, has been demonstrated in the context of the MLKL 4HB death domain in a previous publication (Hildebrand et al., 2014). These points have been more clearly outlined in the text **(P8, lines 8-19)***

Hildebrand JM, Kauppi M, Majewski IJ, Liu Z, Cox AJ, Miyake S, Petrie EJ, Silk MA, Li Z, Tanzer MC *et al* (2020) A missense mutation in the MLKL brace region promotes lethal neonatal inflammation and hematopoietic dysfunction. *Nat Commun* 11: 3150

Hildebrand JM, Tanzer MC, Lucet IS, Young SN, Spall SK, Sharma P, Pierotti C, Garnier JM, Dobson RC, Webb AI *et al* (2014) Activation of the pseudokinase MLKL unleashes the four-helix bundle domain to induce membrane localization and necroptotic cell death. *Proc Natl Acad Sci U S A* 111: 15072-15077

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1st Revision - Editorial Decision 14th Jan 2021

Dear John,

Thank you for submitting your revised manuscript. Your study has now been reassessed by the original reviewers, whose reports are enclosed below.

As you can see, referee #2 finds that the revisions have very much improved the quality of your study. However, this reviewer and referee #1 also have remaining points that need to be fixed before they can recommend the manuscript for publication here.

Therefore, we invite you to address the last pending issues as requested by the referees.

Feel free to contact me if you have any questions about the submission of the revised manuscript to The EMBO Journal. I thank you again for the opportunity to consider this work for publication and look forward to receiving your revision.

Best regards,

Elisabetta

Elisabetta Argenzio, PhD
Editor
The EMBO Journal

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Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

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- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
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Referee #1:

The MLKL-USP21 fusions used in Fig. 7 are central to the notion that mono-ubiquitination of MLKL keeps necroptosis in check by promoting degradation of MLKL. Yet Fig. 7B and 7D don't fully convince me that MLKL-USP21(CR) is ubiquitinated, whilst MLKL-USP21 is not. The fusion proteins are double the size of wild-type MLKL, which makes detecting Ub banding patterns with the MLKL antibody difficult. In addition, if MLKL-USP21 isn't ubiquitinated then what ubiquitinated protein is it interacting with to be pulled down by GST-UBA so effectively? To circumvent this issue, can the authors show ubiquitination definitively by pull-downs that use SDS- or urea-denatured lysates? Such conditions should prevent pull-down of MLKL indirectly through its binding to ubiquitinated proteins.

Minor points re figure labeling:
Fig. 2C - MLKL is running below 50 kDa, whereas it runs at 50 kDa in all other panels.
Fig. 5C - label is ph-MLKL, whereas pMLKL is used in all other panels.

Referee #2:

The authors now provide evidence about the mechanism of MLKL specific ubiquitination in necroptosis and a possible biological relevance. They show that MLKL ubiquitination is dependent on its oligomerization and association with crude membrane fractions, but does not require phosphorylation. Based on data obtained with mouse and human orthologs they suggest that MLKL ubiquitination is relevant to restrict MLKL autoactivation to avoid errant necroptosis.
While it is a pity that some of the experiments could not be performed due to the current pandemic, the manuscript has notably improved. A couple of issues should still be solved, though.

Major:

-The authors should solve the issues with the lack of NSA inhibition, as this is an important control. The fact that NSA does not block TSI induced necroptosis at high levels of exogenous MLKL expression is surprising as this is a well-established inhibitor of necroptosis used in the field also under overexpression conditions.
-The Incucyte experiments should be presented in a way that include the statistics of the repetitions carried out for each experiment, for example by plotting the average curves of cell death induction over time.
- In line 15, the authors claim that ubiquitylation appears to occur around the same time as membrane permeabilization (based on PI measurements) but they didn't follow the kinetics of ubiquitination as they did for PI positive cells. In fact, if this is a regulatory mechanism, I would expect that ubiquitination takes place before cell death. Authors should clarify this aspect.

Minor:
- The authors nicely propose that MLKL autoactivation is restricted by ubiquitination. However, this conclusion is based on results obtained with intrinsically active mutants or fusion versions of the protein. I am still wondering which would be the possible mechanisms of WT MLKL autoactivation of in cells. They should discuss possibilities.

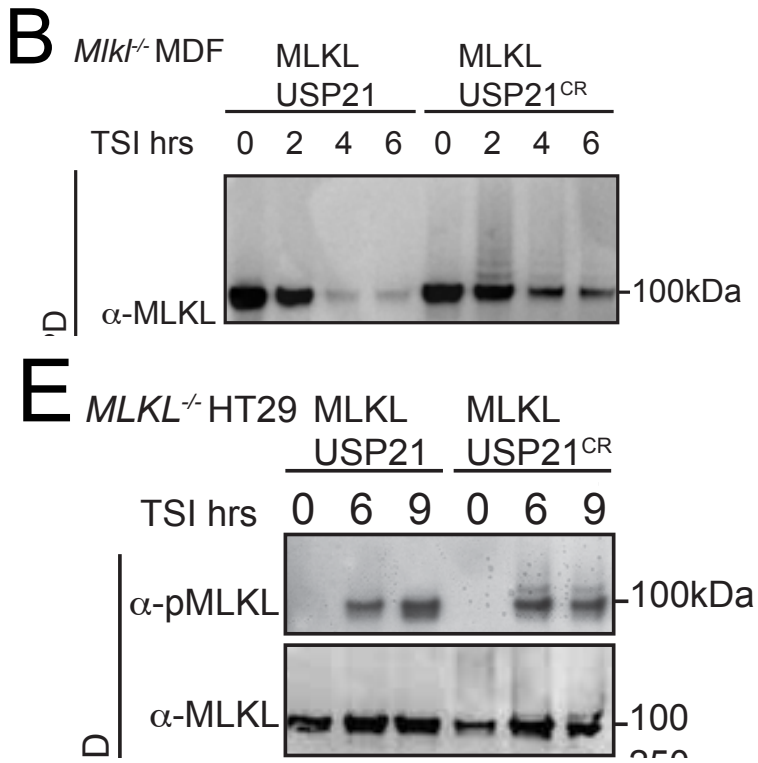
Referee #1:

The MLKL-USP21 fusions used in Fig. 7 are central to the notion that mono-ubiquitination of MLKL keeps necroptosis in check by promoting degradation of MLKL. Yet Fig. 7B and 7D don't fully convince me that MLKL-USP21(CR) is ubiquitinated, whilst MLKL-USP21 is not. The fusion proteins are double the size of wild-type MLKL, which makes detecting Ub banding patterns with the MLKL antibody difficult. In addition, if MLKL-USP21 isn't ubiquitinated then what ubiquitinated protein is it interacting with to be pulled down by GST-UBA so effectively? To circumvent this issue, can the authors show ubiquitination definitively by pull-downs that use SDS- or urea-denatured lysates? Such conditions should prevent pull-down of MLKL indirectly through its binding to ubiquitinated proteins.

The reviewer's suggestion is a good one to address whether the MLKL fusion is ubiquitylated and we have therefore done our best to address the thought behind it experimentally. Nevertheless, before we discuss that, we would also like to stand our ground a little with regard to the data in **Fig. 7B** and **E** (shown as **Response Figure 1.**). Throughout the manuscript we have shown in the non-fusion MLKL a pattern of ubiquitylation (confirmed in a number of ways) that looks very similar in the CR mutant but is not present in the wild type USP21 fusion. The levels of the two fusion proteins are very similar, so direct comparisons are possible, and while the laddering could be due to other modifications, Occam's razor would suggest that this is indeed ubiquitin modification.

Regarding our experimental response, and, incidentally, thanks for understanding the difficulty of showing clear laddering on high MW bands, we have tried several times using either SDS or urea in the lysis buffer, yet both made the whole pull-down less efficient. Since the banding is already difficult to detect (as the reviewer acknowledges) this was not successful. Therefore, we tried another approach and incubated the UBA-PD fractions with recombinant USP21. The expectation is that this DUB treatment post UBA pulldown will collapse the high Mw laddering in the MLKL-USP21^{CR} samples, which it does (**Sup.Fig.7C**). Furthermore, by collapsing the high MW species, it becomes very clear that we enrich for MLKL-USP21^{CR} mutant upon TSI treatment compared to both non-TSI treated and compared to MLKL-USP21. Indeed there is no enrichment in the MLKL-USP21 samples ± TSI. Unfortunately, we still do not have a clear answer as to why we precipitate MLKL-USP21 at all with UBA, although we note that we

consistently pulldown both WT mouse and human MLKL in the absence of TSI stimulation and therefore without obvious ubiquitylation of MLKL (E.g. **Fig. 5C-E, 6B, 7B, E**).



Response Figure 1. Magnified regions of Fig. 7 B&E

We have altered the text as follows at ***p11 line 1-2***:
"These high MW MLKL-USP21^{C221R} species were reduced and collapsed into non-ubiquitylated form by digestion of recombinant USP21 ***(Supp. Fig. 7C)****."*

Minor points re figure labeling:

Fig. 2C - MLKL is running below 50 kDa, whereas it runs at 50 kDa in all other panels.
Fig. 5C - label is ph-MLKL, whereas pMLKL is used in all other panels.

We have corrected these issues – thanks for pointing them out.

Referee #2:

The authors now provide evidence about the mechanism of MLKL specific ubiquitination in necroptosis and a possible biological relevance. They show that MLKL ubiquitination is dependent on its oligomerization and association with crude membrane fractions, but does not require phosphorylation. Based on data obtained with mouse and human orthologs they suggest that MLKL ubiquitination is relevant to restrict MLKL autoactivation to avoid errant necroptosis.

While it is a pity that some of the experiments could not be performed due to the current pandemic, the manuscript has notably improved. A couple of issues should still be solved, though.

Major:
-The authors should solve the issues with the lack of NSA inhibition, as this is an important control. The fact that NSA does not block TSI induced necroptosis at high levels of exogenous MLKL expression is surprising as this is a well-established inhibitor of necroptosis used in the field also under overexpression conditions.

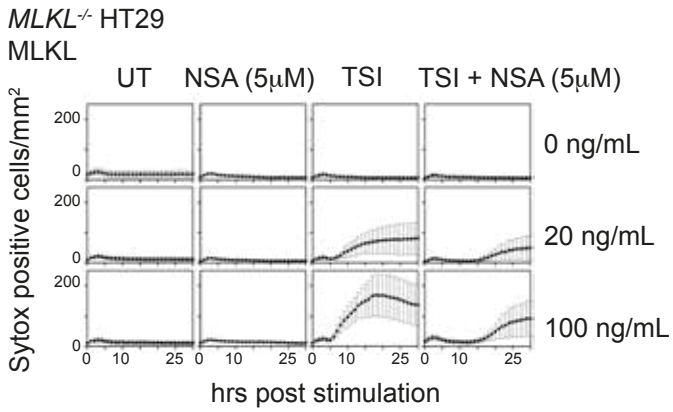
In our new **Fig. 7D**, we have shown that expression of MLKL, MLKL-USP21, MLKL-USP21^{CR} in *MLKL*$^{-/-}$ HT29 cells can induce necroptosis followed by TSI, which can be delayed by 1 µM NSA. This delay is notable when comparing cell death at 10-hr timepoint (labelled with red dash lines in the figure).

We tried to increase the NSA to 5 µM to see if more significant inhibition can be detected against only WT MLKL (see our **Response Figure 2** below). While 5 µM NSA did indeed extend the delay caused by 1µM in cell death from 10 to 15 hrs, this inhibition too was overcome, indicating that NSA can only block MLKL-induced cell death within a certain threshold.

While other authors have used NSA in over expression systems, one difference in our setup is that MLKL is being constantly induced throughout the experiment. Since NSA blocks MLKL execution by forming a covalent bond at Cys86 (Sun *et al*, 2012), and since increased concentrations extend the delay, it seems reasonable to believe that over time levels of free NSA are reduced (perhaps since it is rather a reactive compound by reacting with other proteins too) and newly produced MLKL can therefore no longer be inhibited.

We have altered the text as follows at ***p11, lines 15-19***:

"NSA was able to delay TSI-induced cell death for all MLKL species, but became less effective over time, presumably overwhelmed by ongoing expression of MLKL. Induced expression of MLKL-USP21 also caused TSI-independent cell death from 16 hrs, without detectable MLKL phosphorylation (t_0 = 25 hrs induction, ***Fig. 7E****), and this could also be delayed by NSA (****Fig. 7D****)."*



Response Figure 2. Increasing NSA to 5 μM further delays TSI-induced cell death in the MLKL overexpression system but cannot prevent it.

The Incucyte experiments should be presented in a way that include the statistics of the repetitions carried out for each experiment, for example by plotting the average curves of cell death induction over time.

We have replaced all Incucyte data with plots of the mean ± SEM and have indicated the number of independent repeats in all cases. The only exception is **Supp. Fig 7B**, where there was no increase in Sytox positivity under any condition at all times (thus error bars are not present nor informative)

In line 15, the authors claim that ubiquitylation appears to occur around the same time as membrane permeabilization (based on PI measurements) but they didn't follow the kinetics of ubiquitination as they did for PI positive cells. In fact, if this is a regulatory mechanism, I would expect that ubiquitination takes place before cell death. Authors should clarify this aspect.

Thank you for this comment. You are correct, we too believe that ubiquitylation is likely to occur before membrane permeabilisation, however there was not the resolution in the assay to allow us to make more than a generic comment at that stage.

To be more accurate we have now stated on ***p5, lines 16-18***:

*"Ubiquitylation probably occurs before or possibly at the same time as membrane permeabilization, because by the time of UBA-PD for MDFs (3 hrs) and HT29 cells (16 hrs), cells were at least 60% propidium iodide (PI)-positive (**Supp. Fig. 1A**)."*

This point is further illustrated at ***p7, lines 19-22***:
"*Ubiquitylated MLKL emerged and accumulated within the whole cell lysate and the crude membrane fraction from 90 minutes post-stimulus onwards, coinciding precisely with the appearance of phosphorylated MLKL and onset of cell death (**Fig. 3A & Supp. Fig. 1A**).*"

Minor:
- The authors nicely propose that MLKL autoactivation is restricted by ubiquitination. However, this conclusion is based on results obtained with intrinsically active mutants or fusion versions of

the protein. I am still wondering which would be the possible mechanisms of WT MLKL autoactivation of in cells. They should discuss possibilities.

We appreciate this point and agree that this should be further addressed for readers. Possibilities include low level activation of MLKL by kinases other than RIPK3 during processes, or low level conformational change to the 'active state' that is independent of phosphorylation we have included these possibilities on ***p14, lines 18-21***:

"*This further supports the idea that ubiquitylation of MLKL is an important 'insurance policy' against low level activation of MLKL by other cellular kinases or low-level spontaneous transition to the active conformation.*"

Notice of minor data removal and figure re-arrangement since last submission.

In the previous versions of manuscript, we carefully discussed our observation that MLKL-USP21 was apparently phosphorylated in the absence of TSI, based on our use of a pMLKL antibody (Abcam ERP9515(2)). We did note however our concern that, while obviously the antibody was relatively specific, we couldn't exclude the possibility that it might cross react with high levels of unphosphorylated MLKL. To clarify this point, we tried a recently developed pMLKL antibody (Cell Signaling#37333)(Samson *et al*, 2021) of improved specificity, and found that with this antibody, MLKL-USP21 does not seem to be phosphorylated without TSI stimulation (shown in **Supp. Fig. 7C**). Hence, we removed the discussion from the text and removed the misleading pMLKL blots from **Fig. 7B** and **Supp. Fig. 7A** and the former **Supp. Fig. 7C** has also been fully removed. The removal of these pMLKL panels does not alter our major findings or discussion points, and in our opinion, substantially clarifies our manuscript for readers. All references to these figures in the manuscript have been updated to reflect these changes, and we are confident that all other pMLKL blots in our manuscript, which were shorter exposures, reflect the specific detection of pMLKL.

References

Samson AL, Fitzgibbon C, Patel KM, Hildebrand JM, Whitehead LW, Rimes JS, Jacobsen AV, Horne CR, Gavin XJ, Young SN *et al* (2021) A toolbox for imaging RIPK1, RIPK3, and MLKL in mouse and human cells. *Cell Death Differ*

Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X *et al* (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell* 148: 213-227

2nd Revision - Editorial Decision 12th Jul 2021

Dear John,

Thank you for submitting your revised study. The manuscript has been sent back to the original referees and we have now obtained their reports, which are appended below for your information.

As you can see, while the referees find that their criticisms have been adequately addressed and recommend the study for publication, reviewer #1 asks you to acknowledge in the text that it is unclear why non-ubiquitylated MLKL is recovered in the UBA-PD experiments.

In addition, there are few editorial issues concerning the text and the figures that I need you to address before we can officially accept your manuscript.

-> List the contribution of Samuel N. Young, Aleksandra Bankovacki, Xiangyi Wang, Michelle Tang, Jason Howitt, Che A. Stafford, Ueli Nachbur, Cheree Fitzgibbon, Sarah E. Garnish, Andrew I. Webb, and James M. Murphy to the "Author contributions" section in the manuscript.

-> Upload the figure singularly (one file/figure).

-> Supplementary Figure 2 is missing, and it is not called out in the main manuscript text. Please check the figure order/number and update them in the main text.

-> Call out Supplementary Figure 3 in the main text.

-> We generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

-> Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

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-> In addition, I would encourage you to provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

I thank you again for giving us the chance to consider your manuscript for publication in The EMBO Journal and look forward to your revision.

Kind regards,

Elisabetta

Elisabetta Argenzio, PhD
Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

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- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 10th Oct 2021.

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

Referee #1:

The authors have made every effort to address the points raised.
I support publication but suggest that they acknowledge in the text that it is unclear why non-ubiquitylated MLKL is recovered in their UBA-PD experiments.

Referee #2:

The authors have addressed adequately the reviewers concerns.

3rd Revision - Editorial Decision 20th Sep 2021

Dear John,

Thank you for submitting your revised manuscript to the EMBO Journal. I have now had a chance to look at everything and all looks good.

I am therefore very pleased to accept the manuscript for publication here.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- 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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs 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 and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship 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 χ2 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.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	not applicable
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No animal studies are included
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For animal studies, include a statement about randomization even if no randomization was used.	No animal studies are included
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No animal studies are included thus unbiased group allocation not required. No other studies involved methods based on subjective scoring.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No animal studies are included
5. For every figure, are statistical tests justified as appropriate?	Statistical testing was not employed. All data are provided as mean +/- standard error, no tests of statistical significance were performed to deomstrate differences between groups on these data.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	not applicable (see #5)

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Is the variance similar between the groups that are being statistically compared?	not applicable (see #5)

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies used have been validated in relevent mouse or human knock-out cell lines. For the most up-to date validation, please consult https://www.biorxiv.org/content/10.1101/2020.10.26.356063v1
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines are routinely screened for mycoplasma contamination in-house.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	No animal studies are included
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	No animal studies are included
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	No animal studies are included

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	No human studies are included
12. 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.	No human studies are included
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	No human studies are included
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	No human studies are included
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	No human studies are included
16. 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.	No human studies are included
17. 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.	No human studies are included

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	A 'Data Availability' section has been added as required, it reads; "The raw mass spectrometric data and the MaxQuant result files have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol, Csordas et al., 2019) partner repository with the dataset identifier: PXD015537.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	see #18
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	not relevant to this study
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	not relevant to this study

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	not relevant to this study