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LUBAC Regulates NF-κB Activation upon Genotoxic Stress by Promoting Linear Ubiquitination of NEMO

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1st Editorial Decision 19 November 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received the reports of three expert reviewers, which you will find enclosed below. I am afraid that these reports do not offer strong support for publication in The EMBO Journal, as you will see. I prefer not to repeat all the individual points of criticisms in detail in this letter, but while the reviewers all acknowledge that the proposed role for LUBAC in genotoxic stress-induced NF-kBa activation would be of interest in principle, they remain unconvinced that the current experimental evidence offers sufficient and definitive support for these conclusions and, importantly, for the physiological significance of the presented results. Given that most of these major concerns appear to be shared by more than one referee, I am afraid that these critical opinions leave me with little choice but to conclude that we will not be able to offer publication of this study in The EMBO Journal. We receive a high number of submissions, and can therefore only afford to accept those few that are met with elevated enthusiasm from at least a majority of referees already upon initial review. In any case, I would like to thank you for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but hope nevertheless that you will at least find our referees' comments helpful.

Yours sincerely,

Editor
The EMBO Journal

Niu et al. present data in support of a role for LUBAC in genotoxic stress-induced NF-kB and TAK1 activation by linearly ubiquitinating NEMO on residues K285 and K309. The authors promote a model in which genotoxic agents induce phosphorylation, mono-ubiquitination and nuclear export of NEMO. After reaching the cytoplasm NEMO associates with LUBAC in an ELKS-dependent manner, becomes poly-ubiquitinated and induces activation of NF-kB and TAK1. In the absence of a functional LUBAC or its ubiquitination sites on NEMO NF-kB and TAK1 signalling are diminished, resulting in decreased genotoxic stress-induced up-regulation of anti-apoptotic genes and higher susceptibility to cell death.

Major points:

1. On the basis of the presented data the authors cannot claim to have shown a physiological role for LUBAC in genotoxic stress-induced NF-kB activation. The majority of the presented data is based on overexpression experiments. Their significance for a physiological role of genotoxic stress-induced, LUBAC-mediated NF-kB activation is rather doubtful. The little data presented on endogenous interactions is not convincing and lacks important controls. In particular, other genotoxic stimuli (apart from TNF as positive control) should be included in Figures 2f and 6d together with a kinetic analysis of when the interaction between NEMO and LUBAC takes place.

2. Another important issue is that the authors base several experiments on findings presented by Tokunaga et al. (Nature Cell Biology, 2009) obtained with TNF. Although important aspects of the presented data are in contradiction to Tokunaga et al. the authors do not comment on these differences. In particular in Figure 1b and 4c, why is TNF-induced NF-kB activity (as determined by EMSA and IKK activity assays) not diminished in HOIL knockout MEFs?

3. The authors suggest that NEMO were the only target of LUBAC. How then do the authors explain that TAK1 activity is also affected by genotoxic stress but not by TNF (Figure 4)? The authors intend to offer an explanation for this discrepancy by suggesting a crosstalk between NF-kB and TAK1, however experimental evidence presented here is insufficient and it is unclear why such a crosstalk would not exist with TNF if it existed under genotoxic stress. To make such a claim, the authors would need to do activity assays such as the ones they performed in NEMO-deficient cells. A related question is whether TAK1 activity is suppressed in the absence of NEMO and genotoxic stress-induced JNK activation also diminished.
Along these lines, why is NEMO attached to TAB2 in unstimulated cells (Figure 5c)? The significance of this experiment is doubtful since it was only done with overexpressed LUBAC and not under endogenous conditions. If the authors want to claim that LUBAC plays a role in genotoxic stress-induced NF-kB activation, overexpression should not be required to observe this effect. The authors also over-interpret their data when they conclude that TAB2 binds to linear ubiquitin chains attached to NEMO. It is well possible that NEMO is also K63-ubiquitinated and that the interaction is mediated via this ubiquitin type of chain linkage. Analyzing the blot with a K63-specific antibody might clarify this issue. What would happen in the same set-up if the K285/K309R NEMO mutant were used? If an interaction with TAB2 were still visible this would be incompatible with the proposed model.

4. The authors claim that the signals detected with anti-ubiquitin antibodies following NEMO pull-down (e.g. in figs 2a-d, 3 and 6) are due to ubiquitin chains attached to NEMO. Why then did the authors not detect any modifications when incubating these blots with an anti-NEMO (or anti-myc) antibody? Most of the blots are cut just below 50 kDa (corresponding to the size of NEMO), even though it seems as if there were more bands below this (e.g. in figs 2d, 3a, b and 6a-c). These data can only be evaluated properly when the entire blots are shown.

5. How sure are the authors that the bands detected for endogenous HOIL-1 and HOIP are indeed the correct ones? HOIL-1 for example is known to appear as a double band. Furthermore, Tokunaga et al. showed that HOIP is destabilized in HOIL-1 ko liver extracts. Is this not true for MEFs? Are the authors sure that they detect the correct band for HOIP (e.g. in Figure 4c)? Showing a HOIP knockdown on the same gel might help to clarify this. It would also be helpful if molecular weight markers were included in all figures.

6. It is unclear how the calculation of the fold induction as determined by phosphoimager was done. Did the authors consider the intensities for Oct-1 when determining the respective fold induction

(e.g. in Figure 1, lane 7, in which Oct-levels are also significantly decreased) or how where they calculated? Some of the presented numbers do not match with the differences observed upon visual inspection of the blots.

7. In general the quality of the blots is rather low and requires major improvement (only as an example, in Fig 3c the input blots are not acceptable).

Minor points:

8. Is the labelling correct in Fig 1c? Was HOIP overexpressed and knocked-down at the same time?
9. A HOIP knock-down should be included in Fig 1e for consistency of the Figure and to show a further read-out that genotoxic stress-induced NF-kB activity was decreased in the absence of LUBAC.
10. If denaturation were sufficient, HOIL-1 and HOIP should not be co-immunoprecipitated with NEMO in Fig 2a. The authors would need to show the blot for LUBAC in the IP (on the same gel as the input). Furthermore the authors claim that less ubiquitin is attached to the NEMO mutant upon etoposide treatment. However there is already less NEMO pulled out which makes this conclusion invalid.
11. What is the band at 72 kDa in the IP in Fig 2b? Why is no smear detectable but only a small band at 170 kDa? This data is not convincing.
12. The authors should repeat the experiment shown in Fig 2c under endogenous conditions, i.e. without overexpression of NEMO. Is there less NEMO-Ub if HOIP is knocked down? This would be more convincing.
13. It has been described that LUBAC interacts with NEMO when all proteins are overexpressed without further stimulation (Tokunaga et al., NCB 2009). It is therefore unclear what the authors intend to show in Fig 2e.
14. In Fig 3c, the cytosolic and nuclear fractions need to be shown on one blot. Otherwise is it difficult to compare the results. Also, the purity of the fractions cannot be judged when the respective markers are on different blots. I am concerned whether the bands supposedly representing ubiquitinated NEMO are indeed what the authors suggest as this would suggest that NEMO is only highly poly-ubiquitinated.
15. Why is the UbS85A NEMO mutant shown in Fig 3d smaller in size despite attachment of a ubiquitin (given that the myc and HA-tag are of approximately the same size)? What happens in this setting when HOIP is knocked down?
16. As a control for Fig 3e it would be helpful to see the same experiment when wt NEMO is overexpressed to show that Ku was indeed functional and able to inhibit ubiquitination of wt NEMO.
17. Is the quantification of the IKK activity normalized to GST levels in Figure 4a? From the expression levels I am concerned whether TNF-induced IKK activity was indeed affected as significantly as suggested by the quantification blot.
18. Please show the expression control blots for Figs 4f and g.
19. In the text the authors cite Komander et al. who showed that TAB2 specifically binds to K63-linked tetra-ubiquitin chains, but was only able to bind to linear ubiquitin chains whne they are longer. Yet In Fig 5 data are presented that suggest an interaction between TAB2 and linear tetra-Ubiquitin. This finding contrasts with the findings by Komander et al. but the authors do not comment on this.
20. The apparent molecular weight difference between Ub2 and Ub3 is quite large. Is it possible that the bands refer to Ub1 and Ub3? Please indicate size markers to clarify this.
21. If the authors want to claim that the NZF of TAB2 were required for binding to linear ubiquitin chains it would be good to show an NZF-only mutant in the same assay.
22. Does NEMO interact with ELKS? An ELKS blot should be shown in the IP in Fig 6d.
23. The figure legend of Fig 6c is not clear. Was LUBAC also overexpressed in this experiment?
24. How is NEMO ubiquitination altered when CYLD is knocked down? Is ubiquitination more pronounced?
25. Why is the Ub-K285/K309R mutant of NEMO not running differently in Fig 7b? It would be good to show the cellular localization of the mutants in the cell in support of the authors' model.
26. Error bars are missing in Fig 7e. As the experiment was only done twice at least one additional repeat is necessary. How susceptible are the cells for death induced by etoposide when LUBAC is knocked down (and NEMO not overexpressed)?

Referee #2 (Remarks to the Author):

Activation of NF-kB after DNA damage can occur through an atypical pathway requiring the nuclear localization of NEMO. However, the mechanism by which NEMO activates the IKK complex after it returns to the cytoplasm is still not clear. Here, the authors investigate a role for linear ubiquitination of NEMO, which, together with the K63 linked ubiquitination of ELKs, are required events for NF-kB activation after DNA damage.

This is an interesting manuscript that characterizes an important component of the pathway activating NF-kB after genotoxic stress. However, there are a number of weaknesses in the manuscript that must be addressed.

Specific concerns

(1) At numerous places in the manuscript (e.g. Fig. 1A & C, Fig. 2C) a HOIP siRNA is used. However, it appears that these experiments are performed without a control siRNA treatment. This important control must be performed for all these experiments. In addition, in Figs. 4A & B although a number of lanes are indicated as 'Mock' it is not clear if these actually contain a control siRNA (as is needed).

(2) In Figure 1E, the effects on NF-kB activity by the LUBAC-CS mutant are weak. One possibility is inhibition of linear ubiquitination merely changes the timing of NF-kB activation after DNA damage. The authors need to perform a time course after DNA damage (with and without LUBAC inhibition) to more accurately ascertain the extent of inhibition seen.

(3) There is, overall, a reliance on over-expression in many figures. What appears to be missing is a convincing experiment, directly showing endogenous LUBAC dependent, inducible linear ubiquitination of endogenous NEMO after DNA damage. Many experiments just look at total ubiquitination. The experiments which comes closest are Fig. 2D but here LUBAC is still over-expressed and the gel quality is very poor.

(4) An important component of the authors model concerns the timing of NEMO linear ubiquitination (Fig. 3A). However the time course in this figure is minimal while as a control for this experiment, the other nuclear modifications of NEMO referred to are not shown.

(5) In Figure 3C the authors seek to show that linear ubiquitination of NEMO is cytoplasmic. However, the experiment is not convincing - it lacks an IgG control, and is performed with a non-specific ubiquitin antibody. Moreover, there seems to be a high level of nuclear NEMO poly ubiquitination (especially given the relative levels of nuclear NEMO) that is also inducible.

(6) Why is the NEMO S85A mutant inducibly ubiquitinated in Fig. 3D but not in Fig. 3D?

(7) In a number of places throughout the manuscript, a K285/309R NEMO mutant is used (see particularly Figure 7). The authors use this to conclude that linear ubiquitination is important. However, these amino acids are also subject to other modifications and so this mutant provides only limited insight into the role of linear ubiquitination and is, in my opinion, overly used in the manuscript and the data produced over interpreted. The dramatic effect on gene expression in Fig. 7D contrasts with the weak effect in Fig. 1D, suggesting that the former effects results from total inhibition of NF-kB signaling seen with this mutant rather than a specific effect on linear ubiquitination of NEMO.

(8) Similar to (2) - the ability of an ubiquitin fused K285/309R NEMO mutant (Figure 7) to restore NF-kB activation after genotoxic stress is taken as evidence of the importance of linear ubiquitination. I do not understand how the authors can draw this conclusion and indeed think it tends to support the opposite conclusion.

(9) At 6.5 pages, the discussion is too long.

Referee #3 (Remarks to the Author):

The authors pursue their analysis of genotoxic stress-induced NF- B activation and report a novel role for the ubiquitin ligase complex LUBAC and for polyubiquitination with linear chains. They demonstrate that LUBAC induces NEMO polyubiquitination with linear chains on Lys 285 and 309, and that this ubiquitination is required for activation of the IKK and TAK1 kinase complexes, and ultimately for NF- B activation. They show that this ubiquitination takes place in the cytoplasm, downstream of the nuclear events that affect NEMO in response to DNA damage. They also show that K63-linked ubiquitination of the adaptor protein ELKS facilitates NEMO linear ubiquitination. These data are important and extend the range of NF- B activating signals that involve linear polyubiquitination events. However some additional data and controls are required before publication can be recommended :
- figure 1A : it would be useful to show a control which is insensitive to downregulation of LUBAC : NF- B activation by IL1 or LPS for example. More generally, using a siRNA targeting HOIP is certainly more convincing than overexpressing a dominant negative form. In figure 1C an 1D, the siRNA should be used instead of LUBAC-CS.
- Figure 1B : it is impossible to compare HOIL+/+ and HOIL-/- cells as they are from a different origin. The experiment should be repeated with HOIL-/- cells complemented by the HOIP cDNA. The same applies to figures 4C and 4D.
- Figure 2B : the polyUb smear in lane 6 is barely visible. A better blot should be provided.
- figure 2D : a further proof that linear polyUb is involved would require cotransfection with ubiquitin K0.
- Figure 4B : there is no effect of the HOIP siRNA on TAK1 activation. Do the authors imply that linear polyubiquitination plays a different role in DNA damage- vs TNF-induced NF- B activation ?
- A general concern : seing an effect with the K285/309 mutation does not necessarily implicate a role for linear ubiquitination. K285 is also a site for monoubiquitination and K309 for sumoylation. This should be acknowledged in the Discussion.
- On page 18 the authors write : 'Then, the linear polyubiquitin chains on NEMO and the K63-polyubiquitin chains on ELKS may serve as platforms for recruiting TAK1 and IKK complexes' This is confusing : i) if NEMO recruits TAK1/TAB2 through linear chains, then ELKS is no longer required. However binding of TAB2 to linear chains is very weak. ii) If both IKK and TAK1 complexes are recruited to ELKS, then linear polyubiquitination is no longer necessary. iii) If IKK/NEMO is recruited to the K63 chains of ELKS, and TAK1 to the linear chains of NEMO, all molecules play a role, but again the affinity of TAB2 for linear chains is quite weak. In fact the only protein that seems to exhibit reasonable affinity for linear chains is NEMO itself. The authors should modify their discussion accordingly.
- The authors should also discuss how the different modifications occur : NEMO is first sumoylated on K277 and 309, then desumoylated and monoubiquitinated on the same Lys residues, and also monoubiquitinated on Lys 285. Then linear polyUb chains are added on Lys 285 and 309 : does it require deubiquitination of Lys 285, or extension of the monoUb by LUBAC ? The situation would be different for Lys 309 where desumoylation would be followed by linear polyubiquitination ?
- Minor point : Figure 2C : what is the intense band in the upper panel ? Immunoglobulins ? MW markers should be included.

Additional correspondence 26 November 2010

Thank you for giving our manuscript an opportunity to be considered by the EMBO J. We also appreciate all three reviewers' time and insightful comments. After we carefully read the reviewers' critiques, we feel we will be able to address most, if not all, of the concerns raised by the reviewers. The major issue about the physiological significance, which was insufficiently supported by our data generated from overexpression conditions, was caused by the limit of the reagents. At the time we performed those experiments, we have not obtained the Linear Ub, HOIL and HOIP antibodies which are not commercially available. With these valuable reagents provided by Dr. Iwai, we are now able to repeat these experiments in untransfected cells, and we are confident those data are repeatable under this setting.

We are wondering, if we are able to address the reviewers' critiques with new experiments and

improved data, whether our manuscript stands a chance of being re-considered by the EMBO J?

Thank you again for your time and giving us the opportunity.

Additional correspondence 26 November 2010

Thank you for your message and your inquiry about the possibility to resubmit an improved version of your recent submission, EMBOJ-2010-76307. I realize that the referee reports indicated that there was interest in principle in your results and conclusions, and would therefore not want to exclude the possibility of looking once more at the study should you really be able to comprehensively and decisively address the important concerns. However I do notice that the experimental issues raised were both very serious and numerous, and therefore hope you understand that I am not in the positions to make any commitments or predictions on the potential chances of such a new manuscript, which would have to be treated as a new submission and only sent back to the referees if we thought that their concerns had be largely addressed, and if the novelty of the message has not been compromised by the time of resubmission.

Yours sincerely,
Editor
The EMBO Journal

Resubmission 21 March 2011

I respectfully enclose the revised version to previous manuscript (EMBOJ-2010-76307) entitled "LUBAC Regulates NF- B Activation upon Genotoxic Stress by Promoting Linear Ubiquitination of NEMO" to be considered for publication in the EMBO Journal. We have revised the manuscript in order to best address the comments of the reviewers. I believe that the input of the reviewers has improved the final version of our manuscript. I provide a point-by-point answer to each of the reviewer's comments.

Referee #1
1. On the basis of the presented data the authors cannot claim to have shown a physiological role for LUBAC in genotoxic stress-induced NF-kB activation. The majority of the presented data is based on overexpression experiments. Their significance for a physiological role of genotoxic stress-induced, LUBAC-mediated NF-kB activation is rather doubtful. The little data presented on endogenous interactions is not convincing and lacks important controls. In particular, other genotoxic stimuli (apart from TNF as positive control) should be included in Figures 2f and 6d together with a kinetic analysis of when the interaction between NEMO and LUBAC takes place.

We provide additional evidence to support our conclusion on physiological significance of LUBAC in genotoxic NF- B activation. Depletion of endogenous HOIP (catalytic subunit of LUBAC) by treatment with specific siRNAs significantly inhibited genotoxic agent-induced NF- B activation (new Figs 1A, 1E, 1G) and NEMO linear ubiquitination (new Fig 2C) in human cells. These results are consistent with our observations in HOIL-1 deficient mouse cells and human cells expressing a dominant negative mutant of LUABC, indicating a critical role of endogenous LUABC in mediating genotoxic NF- B activation. As suggested by the reviewer, we also conducted additional co-IP experiments using different timepoints as well as various genotoxic drugs and these data are shown as new Figs 2G and 2H. These new data clearly showed that endogenous LUBAC:NEMO association can be induced by different genotoxic stimuli (CPT, Dox, Etop, Figs 2G-H, 6D) with a delayed kinetics (Fig 2G). As additional controls, we include IP performed on untreated sample as well as IP with control IgG. Moreover, the critical role of LUBAC in regulating cellular response to genotoxic stress was further supported by our new data in Fig 7E showing that depletion of HOIP significantly increased genotoxic drug-induced apoptosis in human cancer cells.

2. Another important issue is that the authors base several experiments on findings presented by Tokunaga et al. (Nature Cell Biology, 2009) obtained with TNF. Although important aspects of the presented data are in contradiction to Tokunaga et al. the authors do not comment on these differences. In particular in Figure 1b and 4c, why is TNF-induced NF-kB activity (as determined by EMSA and IKK activity assays) not diminished in HOIL knockout MEFs?

TNF is a very strong activator of NF- B signaling, when compared to genotoxic stress. We used treatment with TNF (10 ng/ml) for 30 min, a relatively high dose, in all of our experiments as a positive control for activation of classical NF- B signaling pathway. As we showed in our previous publication (Mol Cell, 40: 78-86, 2010), TNF -induced NF- B activation under these experimental conditions is relatively resistant to interference of several critical signaling molecules, such as TAK1 and ELKS, which play important roles in TNF -induced NF- B signaling pathway. We repeated several experiments and replaced Fig 1B (now 1C) and Fig 4C with more representative data, and show a mild but reproducible decrease in TNF -induced activation of IKK (Figs 4A and 4C) and NF- B activation (Figs 1A, 1C, 1E and 1G) in cells where LUBAC function is disrupted biochemically or genetically. These data are in agreement with the results of Tokunaga et al., although we cannot rule out the possibility that TNF could also activate NF- B in a LUBAC-independent fashion under our experimental conditions.

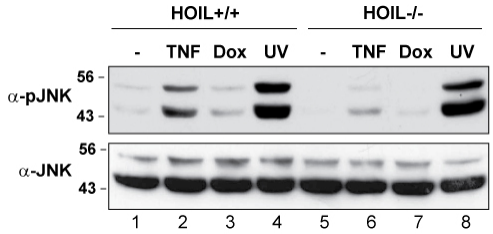
3A. The authors suggest that NEMO was the only target of LUBAC. How then do the authors explain that TAK1 activity is also affected by genotoxic stress but not by TNF (Figure 4)? The authors intend to offer an explanation for this discrepancy by suggesting a crosstalk between NF-kB and TAK1, however experimental evidence presented here is insufficient and it is unclear why such a crosstalk would not exist with TNF if it existed under genotoxic stress. To make such a claim, the authors would need to do activity assays such as the ones they performed in NEMO-deficient cells. A related question is whether TAK1 activity is suppressed in the absence of NEMO and genotoxic stress-induced JNK activation also diminished.

Only a handful of LUBAC substrates have been described, including PKC, NEMO and TRIM25. NEMO was found to be linearly ubiquitinated by LUBAC in response to TNF treatment, which plays a non-degradative role in mediating NF- B signaling. With the expanding function of LUBAC revealed in mediating NF- B activation in response to TNF , CD40, and genotoxic treatments, it is possible that additional LUBAC substrates will emerge by further delineating NF- B signaling pathways. In the current study, we detected a reproducible interaction between NEMO linear ubiquitin chain and TAB2 in cells exposed to genotoxic treatment (Fig. 5D), and we found DNA damage-induced TAK1 activation was significantly diminished in LUBAC-depleted cells (HOIP knockdown or HOIL-deficient), upon genotoxic treatment but not by TNF stimulation (Fig 4B and 4D). We believe that the interaction between TAB2 and NEMO linear ubiquitin chain should also exist in cells treated with TNF . However, the functional significance of such an interaction is relatively less critical in mediating TAK1 activation by a strong NF- B activator such as TNF . Treatment with TNF induces quick and robust ubiquitination of RIP1 and TRAF2, which may be sufficient for TAK1 activation and subsequent IKK activation. However, genotoxic stress-induced NF- B signaling is relatively weak and with a delayed kinetics, which may require LUBAC-dependent NEMO ubiquitination for optimal TAK1 activation. The data of the differential impact of LUBAC on TAK1 activation is consistent with our observation that loss of LUBAC function results in a more significant inhibition of IKK and NF- B activation by genotoxic agents when compared to that by TNF (Figs.1 and Fig4).

In our recent publication (Mol Cell, 40: 78-86, 2010) we provide evidence that NEMO is indispensible for genotoxic agent-induced TAK1 activation, which is dependent on NEMO-mediated ATM nuclear exportation and subsequent ELKS ubiquitination. In this manuscript, we provided an additional mechanistic link between NEMO and TAK1 activation in response to DNA damage. NEMO could also facilitate TAK1 activation by anchoring linear ubiquitin chains that could serve as scaffolding platform for the TAK1 complexes, leading to optimal TAK1 activation. We believe NEMO linear ubiquitination may play a more critical role in achieving NF- B activation in response to relatively a weak activator such as genotoxic stress, when compared to a strong NF- B signaling as TNF .

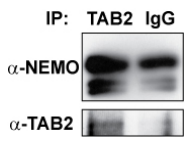
In response to the reviewer's question, we examined whether JNK activation, which also depends on TAK1 kinase activity, is affected in HIOL-1-/- MEFs. As shown below, Abrogation of LUBAC-

dependent NEMO linear ubiquitination did inhibit JNK activation induced by Dox (lane 7 vs. Lane 3), but not that by UV (lane 8 vs. Lane 4). To our surprise, the TNF-induced JNK activation was significantly inhibited in HOIL-1-/- MEFs, indicating HOIL-1 or LUBAC may play a critical role in regulating TAK1-dependent JNK activation by TNF as well. The underlying mechanism still remains to be further investigated, but we hypothesize that it may involve LUBAC-dependent regulation of TAK1 activity.



3B. Along these lines, why is NEMO attached to TAB2 in unstimulated cells (Figure 5c)? The significance of this experiment is doubtful since it was only done with overexpressed LUBAC and not under endogenous conditions. If the authors want to claim that LUBAC plays a role in genotoxic stress-induced NF-kB activation, overexpression should not be required to observe this effect. The authors also over-interpret their data when they conclude that TAB2 binds to linear ubiquitin chains attached to NEMO. It is well possible that NEMO is also K63-ubiquitinated and that the interaction is mediated via this ubiquitin type of chain linkage. Analyzing the blot with a K63-specific antibody might clarify this issue. What would happen in the same set-up if the K285/K309R NEMO mutant were used? If an interaction with TAB2 were still visible this would be incompatible with the proposed model.

Following the reviewer's suggestion, we repeated the experiments as described in the original Fig 5C under endogenous conditions, which are now shown as Fig 5D. We were able to detect association between endogenous TAB2 and linearly ubiquitinated NEMO upon genotoxic stimulation. We also used K63-specific ubiquitin antibody for blotting, but we did not observe convincing NEMO modification by K63-ubiqutin chain. Although we cannot rule out the possibility that NEMO may be modified with K63-chain at a level below the limit of K63-Ub antibody detection, our data support that TAB2 associated with NEMO linear ubiquitin chains in response to genotoxic stimulation. We have repeated this experiment for several times and consistently more NEMO and its modified form (linear ubiquitination) were immunoprecipitated by TAB2 in etoposide-treated cells compared to that in untreated cells, although we still observed NEMO co-IPed with TAB2 in untreated cells. This basal interaction may be due to the large amount of cell lysates used for this sequential IP experiments (similar signal was seen in IgG control IPed sample, please see data below), but the increased association between TAB2 and NEMO/linear ubiquitinated NEMO was only detected in genotoxic drug-treated samples. We show that NEMO linear ubiquitination is significantly diminished in NEMO K285/309R mutant expressing cells (Fig 2), and we did not detect the increased interaction between TAB2 and NEMO K285/309R mutant upon genotoxic stress (data not shown), further supporting a critical role of linear ubiquitin chain in mediating TAB2:NEMO association by genotoxic stress.



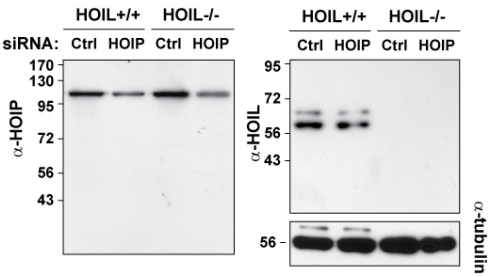
4. The authors claim that the signals detected with anti-ubiquitin antibodies following NEMO pull-down (e.g. in figs 2a-d, 3 and 6) are due to ubiquitin chains attached to NEMO. Why then did the authors not detect any modifications when incubating these blots with an anti-NEMO (or anti-myc) antibody? Most of the blots are cut just below 50 kDa (corresponding to the size of NEMO), even though it seems as if there were more bands below this (e.g. in figs 2d, 3a, b and 6a-c). These data can only be evaluated properly when the entire blots are shown.

We believe that linear ubiquitination only takes place on a small population of NEMO molecules, which is exported from the nucleus, in cells exposed to genotoxic stress. The attachment of polyubiquitin chain to NEMO provides multiple epitopes for anti-ubiquitin antibody recognition, which facilitates the detection of modified forms of NEMO. However, no matter how long the polyubiquitin chain is attached, the NEMO molecule still only provides one epitope for NEMO antibody recognition. Without further enrichment, the level of ubiquitinated NEMO may be still below the threshold of detection by anti-NEMO antibody. We were able to observe a high MW smear signal by NEMO antibody in TAB2-precipitated NEMO sample upon Etoposide treatment (Fig 5D), which can be overlapped to the smear signal detected by anti-linear ubiquitin antibody, supporting the linear ubiquitination of NEMO by genotoxic stress. As suggested by the reviewer, we now show the entire immunoblots in Figs 2B, 2C, 2D, 2E, 2F, 3A, 3C, 3E and 5D.

5. How sure are the authors that the bands detected for endogenous HOIL-1 and HOIP are indeed the correct ones? HOIL-1 for example is known to appear as a double band. Furthermore, Tokunaga et al. showed that HOIP is destabilized in HOIL-1 ko liver extracts. Is this not true for MEFs? Are the authors sure that they detect the correct band for HOIP (e.g. in Figure 4c)? Showing a HOIP knockdown on the same gel might help to clarify this. It would also be helpful if molecular weight markers were included in all figures.

We are confident that the signals we detected represent endogenous HOIP and HOIL-1 proteins by using the respective antibodies for HOIP and HOIL-1, which were developed in Dr. Iwai's laboratory and used in several previous publications from his lab (EMBO J 2006, BBRC 2006, Nat Cell Biol 2009). We did detect a double-band signal using fresh HOIL antibody, as shown in Figs 1C, 4C, 4D and suppl-Fig 1D. However, the top/minor band of double-band signal became less sensitive to reused HOIL-1 antibody.

As shown in Dr. Iwai's publication (BBRC 2006, 351: 340-347, Fig 3E), they detected a similar amount, if not more, of HOIP protein in HOIL-1-deficient MEFs compared to wild type cells using the same antibody, indicating the stability of HOIP in fibroblasts may be less sensitive to HOIL-1 deficiency, compared to that in hepatocytes. We did observe a slightly increase of HOIP level in HOIL-1-deficient MEFs reconstituted with exogenous HOIL-1 (Fig 1D), which is in line with the function of HOIL-1 to stabilize HOIP. We have shown the HOIP antibody was able to detect the decrease of HOIP protein in response to specific siRNA in multiple figures (Figs 1A, 1E, 4A and 4B), and we have add molecular weight marker in these figures to help evaluate the data following the reviewer's suggestion. To further convince the reviewer, we performed HOIP knockdown experiment in wild type and HOIL-1-/- MEF cells (shown below). Either HOIL-1 or HOIP antibody only detect one prominent band in whole cell lysates extracted from cells.



6. It is unclear how the calculation of the fold induction as determined by phosphoimager was done. Did the authors consider the intensities for Oct-1 when determining the respective fold induction (e.g. in Figure 1, lane 7, in which Oct-levels are also significantly decreased) or how where they calculated? Some of the presented numbers do not match with the differences observed upon visual inspection of the blots.

We regret the confusion about how we quantified the EMSA data in Methods section and figure legends. All the EMSA data were quantitated by phosphoimager. The intensity of NF- B activation

band was first normalized to the corresponding Oct-1 signal, then the normalized value were used to calculate fold induction with the untreated sample set as 1. This description has been added into methods section. We also repeated several experiments and now present more representative figures.

7. In general the quality of the blots is rather low and requires major improvement (only as an example, in Fig 3c the input blots are not acceptable).

We repeated a large portion of experiments to obtain better quality blots, and we have replaced a number of figures with more representative data. We hope the reviewer find the quality of our new data, such as new Fig 3C, improved.

8. Is the labelling correct in Fig 1c? Was HOIP overexpressed and knocked-down at the same time?

In our original Fig 1c, we did transfect exogenous HOIP and siRNAs targeting HOIP at the same time. To reduce the complexity of this figure, we have repeated this experiment and present the data in two separate panels as supplemental Figs 1A and 1B. Our new data are consistent with the original results.

9. A HOIP knock-down should be included in Fig 1e for consistency of the Figure and to show a further read-out that genotoxic stress-induced NF-kB activity was decreased in the absence of LUBAC.

Following the reviewer's suggestion, we now include data from HOIP-knock-down cells as Fig 1G. Knock-down of HOIP also significantly inhibited B-reporter's activity by genotoxic stimulation.

10. If denaturation were sufficient, HOIL-1 and HOIP should not be co-immunoprecipitated with NEMO in Fig 2a. The authors would need to show the blot for LUBAC in the IP (on the same gel as the input). Furthermore the authors claim that less ubiquitin is attached to the NEMO mutant upon etoposide treatment. However there is already less NEMO pulled out which makes this conclusion invalid.

We have repeated the experiment and show the improved data as Fig 2A, which support our conclusion.

11. What is the band at 72 kDa in the IP in Fig 2b? Why is no smear detectable but only a small band at 170 kDa? This data is not convincing.

The band below the 72 KD marker was IgG heavy chain (on 7.5% gel) in original Fig 2B. In response to reviewer's comment, we repeated the experiment and showed data with better quality in Fig 2E. We now use a light chain-only secondary antibody, so only light chain can be seen at bottom of the blot.

12. The authors should repeat the experiment shown in Fig 2c under endogenous conditions, i.e. without overexpression of NEMO. Is there less NEMO-Ub if HOIP is knocked down? This would be more convincing.

A new figure 2C panel has been added to show DNA damage-induced endogenous NEMO linear ubiquitination is suppressed in HOIP-depleted cells. We also repeated the experiments in Fig 2C using anti-linear ubiquitin antibody in cells transfected with control siRNA or siRNAs targeting HOIP. This new data are shown in revised Fig 2D.

13. It has been described that LUBAC interacts with NEMO when all proteins are overexpressed without further stimulation (Tokunaga et al., NCB 2009). It is therefore unclear what the authors intend to show in Fig 2e.

Since we have obtained evidence that endogenous NEMO can interact with LUBAC in cells treated by genotoxic agents, we have removed the original Fig 2E, and replaced it with new data showing the kinetics (Fig 2G) of endogenous NEMO:LUBAC association upon treatment with Dox, as well as by other genotoxic drugs (Fig 2H).

14. In Fig 3c, the cytosolic and nuclear fractions need to be shown on one blot. Otherwise is it difficult to compare the results. Also, the purity of the fractions cannot be judged when the respective markers are on different blots. I am concerned whether the bands supposedly representing ubiquitinated NEMO are indeed what the authors suggest as this would suggest that NEMO is only highly poly-ubiquitinated.

We repeated the experiment and now we present the data as the reviewer suggested. Our data strongly indicate that NEMO linear ubiquitination by genotoxic stress takes place in cytoplasm. As our response to major comment 4, longer ubiquitin chain increases Ub-antibody recognition site, so NEMO molecules attached with long polyubiquitin chains are easier to be detected by anti-Ub antibody and yield stronger signals. However, this cannot be interpreted as NEMO is only modified with long ubiquitin chain.

15. Why is the UbS85A NEMO mutant shown in Fig 3d smaller in size despite attachment of ubiquitin (given that the myc and HA-tag are of approximately the same size)? What happens in this setting when HOIP is knocked down?

The Ub-NEMO-S85A construct is tagged with 2xHA, while NEMO-WT/S85A constructs are tagged with 6xMyc (as indicated in Figure legend). Similar results from these plasmids have been shown in our previous publication (Wu et al. Science, 2006, 301:1141-6, Fig 3D). Due to technical issue, we were unable to knock down HOIP by transfecting siRNAs into NEMO-WT and -mutant cells in the current experimental setting. The 1.3E2 cell line used in this experiment can only be transfected by electroporation with low efficiency (roughly <5% total cell population). All NEMO-reconstituted 1.3E2 cells used here are stable clones selected from electroporated cells.

16. As a control for Fig 3e it would be helpful to see the same experiment when wt NEMO is overexpressed to show that Ku was indeed functional and able to inhibit ubiquitination of wt NEMO.

We repeated the experiment in mouse NEMO-reconstituted 1.3E2 cells and now include data from NEMO-WT expressing cells. Our data support that ATM kinase activity is required for DNA damage-induced linear ubiquitination of NMEO-WT, but not Ub-NEMOS85A mutant.

17. Is the quantification of the IKK activity normalized to GST levels in Figure 4a? From the expression levels I am concerned whether TNF-induced IKK activity was indeed affected as significantly as suggested by the quantification blot.

For IKK kinase assay, 1 ug of recombinant GST-I B protein was added into each in vitro kinase reaction as substrate. We did quantify GST levels with ImageJ and used to normalize IKK activity. To address the reviewer's concern, we repeated the experiment and presented a better representative figure in Fig 4A, which show the input GST- I B protein level.

18. Please show the expression control blots for Figs 4f and g.

Blots showing NEMO, IKK and TAK1 expression level have been added in Figs 4F and 4G, respectively.

19. In the text the authors cite Komander et al. who showed that TAB2 specifically binds to K63-linked tetra-ubiquitin chains, but is only able to bind to linear ubiquitin chains when they are longer. Yet In Fig 5 data are presented that suggest an interaction between TAB2 and linear tetra-Ubiquitin. This finding contrasts with the findings by Komander et al. but the authors do not comment on this.

Currently we do not have solid explanation for the discrepancy of the in vitro TAB2:ubiquitin binding results between our lab and the Komander Lab. We reproducibly observed interaction between TAB2 and tetra- even tri-ubiquitin from the linear Ub2-7 mixture we purchased from BioMol, which is different to the purified linear tetra-ubiquitin used by Komander et al. We reasoned that the longer chain (such as Ub7) included in our linear ubiquitin mixture may affect the affinity of TAB2 and facilitates the interaction between TAB2 and shorter chains (Ub3 and Ub4), which requires further investigation.

20. The apparent molecular weight difference between Ub2 and Ub3 is quite large. Is it possible that the bands refer to Ub1 and Ub3? Please indicate size markers to clarify this.

We used 12.5% PAGE gel for these experiments and the ubiquitin signals are consistent with product data sheet provided by manufacturer. We have included molecular weight markers in Figs 5B and 5C.

21. If the authors want to claim that the NZF of TAB2 were required for binding to linear ubiquitin chains it would be good to show an NZF-only mutant in the same assay.

Following this suggestion, we generated TAB2 NZF-only recombinant protein and performed in vitro binding assay. As shown in Fig 5C, TAB2-NZF domain is sufficient for linear ubiquitin interaction.

22. Does NEMO interact with ELKS? An ELKS blot should be shown in the IP in Fig 6d.

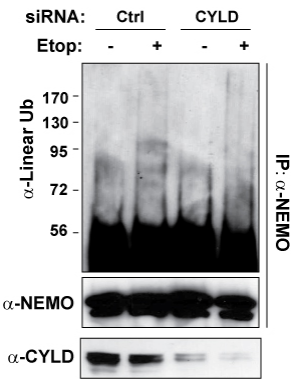
Following the reviewer's suggestion, we repeated the experiment in Fig 6D using additional genotoxic drugs CPT and Dox. ELKS can be detected in NEMO-associated immunocomplex upon genotoxic stress. Our new data further support that genotoxic treatment can induce interaction between NEMO and HOIP in an ELKS-dependent manner. The Fig 6D is replaced with new data and the original panel has been moved to supplementary data as Suppl-Fig 3C.

23. The figure legend of Fig 6c is not clear. Was LUBAC also overexpressed in this experiment?

Both Fig 6C and 6A were performed under endogenous condition without transfection of LUBAC.

24. How is NEMO ubiquitination altered when CYLD is knocked down? Is ubiquitination more pronounced?

We examined the NEMO linear ubiquitination by Etop treatment in HEK293 cells transfected with control siRNA or siRNAs against CYLD (see below). We detected a very subtle, but reproducible, increase of NEMO linear ubiquitination in CYLD-depleted cells. We reasoned that the dramatic decrease of NEMO linear ubiquitination we observed in Fig 6E was due to forced overexpression of exogenous CYLD. However, endogenous CYLD-mediated dismantlement of NEMO linear ubiquitin chain may require an additional regulatory mechanism to liberate CYLD activity to terminate genotoxic NF- B signaling, which may not be activated at the time point (2h after Etop treatment) we used to detect NEMO linear ubiquitination. That may in part explain why we did not observe dramatic increase of NEMO linear ubiquitination in CYLD-depleted cells.



25. Why is the Ub-K285/K309R mutant of NEMO not running differently in Fig 7b? It would be good to show the cellular localization of the mutants in the cell in support of the authors' model.

Ub-NEMO K285/309R mutant is tagged with 3xMyc and NEMO-WT/K285/309R constructs are fused with 6xMyc tag, which results in the similar size of these proteins. We have revised the figure

legend to reflect the difference between constructs.

Following the reviewer's suggestion, we did NEMO subcellular localization staining in HT1080 cells transfected with Myc-NEMO-WT, NEMO-K285/309R or Ub-NEMO-K285/309R mutant using anti-NEMO antibody. As shown in suppl-Fig4C, Cells transiently transfected with NEMO-WT or NEMO-K285/309R showed both nuclear and cytoplasmic staining of NEMO, which is consistent with our previous observation. In contract, Ub-NEMO-K285/309R mutant primarily localized in cytoplasmic compartment, which mimicked our previous observation using Ub-NEMO-S85A mutant (Wu et al. Science, 2006) and another previous report using p53-Ub (Li et al. Science, 2003, 302:1972-5), indicating a role of mono-ubiquitin fusion in directing protein cytoplasmic localization. These results support our model that linear ubiquitination of NEMO is a cytoplasmic event.

26. Error bars are missing in Fig 7e. As the experiment was only done twice at least one additional repeat is necessary. How susceptible are the cells for death induced by etoposide when LUBAC is knocked down (and NEMO not overexpressed)?

Following the reviewer's comment, we performed one additional experiment as in original 7E and pooled data from all three experiments and present this data in suppl-Fig 4E with error bars. We also performed similar experiments using HT1080 cells transfected with control or HOIP siRNAs and examined cells' susceptibility to etoposide-induced apoptosis. As shown in revised Fig 7E, the percentage of apoptotic cells (PI-/Annexin V+) significantly increased in HOIP-depleted HT1080 cells treated with etoposide compared to that in cells transfected with control siRNA, further indicating a critical role of LUBAC in regulating cell apoptosis upon genotoxic stimulation.

Referee #2:

(1) At numerous places in the manuscript (e.g. Fig. 1A & C, Fig. 2C) a HOIP siRNA is used. However, it appears that these experiments are performed without a control siRNA treatment. This important control must be performed for all these experiments. In addition, in Figs. 4A & B although a number of lanes are indicated as 'Mock' it is not clear if these actually contain a control siRNA (as is needed).

We have obtained a standard control siRNA from Dharmacon (siGENOME Non-Targeting siRNA #2 D-001210-02-05) and used it as negative control to repeat all the experiments involved HOIP knock down (Fig 1A, 1C, 2C, 4A and 4B) in our original manuscript, and performed several new experiments using same control siRNA and HOIP-siRNA (shown in Figs 1, 2 and 7). Our data from repeated experiments are consistent with our conclusion that LUBAC plays a critical role in mediating genotoxic NF- B activation by promoting NEMO linear ubiquitination.

(2) In Figure 1E, the effects on NF-kB activity by the LUBAC-CS mutant are weak. One possibility is inhibition of linear ubiquitination merely changes the timing of NF-kB activation after DNA damage. The authors need to perform a time course after DNA damage (with and without LUBAC inhibition) to more accurately ascertain the extent of inhibition seen.

We have performed additional time course experiments using wild type and HOIL-1-/- MEF cells. As shown in suppl-Fig 1D, the disruption of LUBAC function significantly diminished CPT-induced NF- B activation in MEFs while no kinetic alteration of the genotoxic NF- B activation was observed. This data suggested that LUBAC-mediated NEMO linear ubiquitination was required for transducing genotoxic NF- B signaling. The weak inhibition of NF- B activation in Fig 1E (now Fig 1H) may reflect that overexpression of LUBAC-CS may only partially inhibit the endogenous LUBAC function. However, we cannot exclude the possibility that an alternative LUBAC-independent genotoxic NF- B signaling pathway may exist, as we observed a mild induction of NF-B activation in HOIL-/- MEFs upon CPT treatment (Suppl-Fig 1D).

(3) There is, overall, a reliance on over-expression in many figures. What appears to be missing is a convincing experiment, directly showing endogenous LUBAC dependent, inducible linear ubiquitination of endogenous NEMO after DNA damage. Many experiments just look at total ubiquitination. The experiments which comes closest are Fig. 2D but here LUBAC is still over-expressed and the gel quality is very poor.

We appreciate the concern of overexpression experiments shared by the reviewers. Following reviewers' suggestion, we now show that, under endogenous condition, genotoxic drug treatment induces NEMO linear ubiquitination (Fig 2B), and this NEMO linear ubiquitination is HOIP-dependent (Fig 2C). We have repeated several experiments using anti-linear ubiquitin antibody (such as Figs 2D and 3C) instead of total ubiquitin antibody. We also repeated the experiments in Fig 2D (now Fig 2F) without overexpression of LUBAC-WT, and improved data were shown.

(4) An important component of the authors model concerns the timing of NEMO linear ubiquitination (Fig. 3A). However the time course in this figure is minimal while as a control for this experiment, the other nuclear modifications of NEMO referred to are not shown.

In response to the reviewer's critique, we added additional time points in the new Fig 3A. To compare the sequential NEMO modification in the same time frame, we also show NEMO sumoylation and mono-ubiquitination along with linear ubiquitination data, which strongly suggests a chronologically sequential order of these three forms of NEMO modification. It also suggests that mono-ubiquitination of NEMO may serve as a cue for LUBAC-dependent linear ubiquitination, which is consistent with a recent report that mono-ubiquitination of TRIM25 promotes its further linear ubiquitination by LUBAC. (Inn et al, Mol Cell. 2011, 41:354-65.)

(5) In Figure 3C the authors seek to show that linear ubiquitination of NEMO is cytoplasmic. However, the experiment is not convincing - it lacks an IgG control, and is performed with a non-specific ubiquitin antibody. Moreover, there seems to be a high level of nuclear NEMO poly ubiquitination (especially given the relative levels of nuclear NEMO) that is also inducible.

We repeated this experiment with linear ubiquitin specific antibody and included IgG control as the reviewer suggested. A better organized figure is now shown. The potential polyubiquitination of NEMO in nucleus in our original figures may due to much longer exposure of nuclear fraction compared to cytoplasmic fraction, which was used to demonstrate nuclear NEMO. Our new data indicate nuclear NEMO linear ubiquitination is minimal compared to cytoplasmic fraction, which strongly support that genotoxic stress-induced NEMO linear ubiquitination takes place primarily in cytoplasm.

(6) Why is the NEMO S85A mutant inducibly ubiquitinated in Fig. 3D but not in Fig. 3D?

We presume that the reviewer had a concern about Fig 3E compared to Fig 3D. We found NEMO S85A mutation blocked its linear ubiquitination, while Ub-fused NEMO S85A mutant was able to be linearly ubiquitinated in response to genotoxic stimulation. In our original Fig 3E (now Suppl-Fig 3A), we transfected HEK293 cells with Ub-NEMO S85A mutant, whose genotoxic stress-induced linear ubiquitination, in contrast to NEMO-WT, was resistant to ATM inhibition. We have repeated this experiment in mouse NEMO-reconstituted 1.3E2 cells without overexpressing LUBAC, and we also included data from NEMO-WT expressing cells shown as new Fig 3E. The data from both human and mouse cell systems are consistent with our conclusion.

(7) In a number of places throughout the manuscript, a K285/309R NEMO mutant is used (see particularly Figure 7). The authors use this to conclude that linear ubiquitination is important. However, these amino acids are also subject to other modifications and so this mutant provides only limited insight into the role of linear ubiquitination and is, in my opinion, overly used in the manuscript and the data produced over interpreted. The dramatic effect on gene expression in Fig. 7D contrasts with the weak effect in Fig. 1D, suggesting that the former effects results from total inhibition of NF-kB signaling seen with this mutant rather than a specific effect on linear ubiquitination of NEMO.

We appreciate the reviewer's insightful comments and we agree the phenotypes from NEMO K285/309R mutant may not only attribute to the deficiency of linear ubiquitination caused by these mutation. We now include phonotypic analyses in HOIP-depleted cells in revised Fig 7. By comparing data from NEMO K285/309R cells (suppl-Figs 4D and 4E) and HOIP-depleted cells (Figs 7D and 7E), we found the results were consistent, but the NEMO K285/309R cells presented much stronger inhibition compared to HOIP-depleted cells, supporting the reviewer's comments that the phenotypes of K285/309R mutant may stem from the deficiency in multiple forms of NEMO

modification. We have modified the manuscript accordingly.

(8) Similar to (2) - the ability of an ubiquitin fused K285/309R NEMO mutant (Figure 7) to restore NF-kB activation after genotoxic stress is taken as evidence of the importance of linear ubiquitination. I do not understand how the authors can draw this conclusion and indeed think it tends to support the opposite conclusion.

The mechanism by which ubiquitin fusion rescued deficiency of genotoxic NF- B activation caused by NEMO K285/309 mutation is not entirely clear. A potential explanation is that the mono-ubiquitin fusion mimics mono-ubiquitination of NEMO (such as on K285) which facilitated LUBAC-dependent NEMO linear ubiquitination, by either promoting NEMO:LUBAC association or serving as a cornerstone for further extension of linear polyubiquitin. A recent publication (Inn et al, Mol Cell. 2011, 41:354-65.) shows a similar finding that mono-ubiquitination of TRIM25 makes it susceptible for LUBAC-dependent polyubiquitination. We also noticed there were some basal linear ubiquitination of NEMO in cells stably expressing Ub-NEMO S85A mutant (Figs 3D and 3E). Additional investigation will be needed to further delineate the underlying mechanisms.

(9) At 6.5 pages, the discussion is too long.

We have revised the discussion section to make it more concise.

Referee #3
1. Figure 1A : it would be useful to show a control which is insensitive to downregulation of LUBAC : NF*k*B activation by IL1 or LPS for example. More generally, using a siRNA targeting HOIP is certainly more convincing than overexpressing a dominant negative form. In figure 1C an 1D, the siRNA should be used instead of LUBAC-CS.

In a previous report (Tokunaga, et al. Nat Cell Biol 2009), LUBAC has been shown to play a critical role in mediating IL-1 -induced NF- B signaling pathway. NF- *k*B activation induced by TNF and IL-1 was suppressed in HOIL-1 knockout mice and cells derived from these mice. We also found LPS induced NF- B activation was mildly decreased in HIOL-1-/- MEFs (Fig 1C) and in 1.3E2 cells expressing NEMO K285/309R mutant (Fig 4E). These data support a critical role of LUBAC in mediating canonical NF- B signaling initiated from IL-1R/TLRs. Since a recent report (Hostager et al, PLoS One. 2010; 5(6):e11380) showed that LUBAC is required for CD40-induced non-canonical NF- *k*B signaling, our data further corroborate a general role of LUBAC in regulation NF- B signaling in response to a wide range of stimulation.

The data from cells overexpressing LUBAC-CS mutant was a shared concern by the reviewers. Following reviewers' suggestion, we had repeated most experiments using HOIP siRNA and now show data from HOIP-depleted cells and HOIP-CS expressing cells side by side (Fig1A/1B, Fig 1E/1F, Fig 1G/1H, s-Fig1A/1B, Fig 7D/s-Fig 4D, Fig 7E/s-Fig 4E). These data are consistent and support our conclusion that LUBAC is a critical mediator for genotoxic NF- B signaling pathway.

2. Figure 1B : it is impossible to compare HOIL+/+ and HOIL-/- cells as they are from a different origin. The experiment should be repeated with HOIL-/- cells complemented by the HOIP cDNA. The same applies to figures 4C and 4D.

The HOIL+/+ and HOIL-/- MEF cells we used were generated from genetically matched littermate. The only genetic variation between these two cell lines is whether the cells express functional HOIP. We now present new data showing that complementation of wild type HOIL-1 functionally rescued the deficiency of genotoxic NF-*k*B signaling in HOIL-1-/- cells (Fig 1D), supporting a critical role of HOIL-1/HOIP complex in genotoxic NF- *k*B activation. We hope the reviewer find these data acceptable and addressed his/her concern.

3. Figure 2B : the polyUb smear in lane 6 is barely visible. A better blot should be provided.

We have repeated the experiment and improved blots have been shown in revised Fig 2E. Please also see our response to comment#11 of reviewer 1.

4. figure 2D : a further proof that linear polyUb is involved would require cotransfection with ubiquitin K0.

We actually did try the experiment using lysine-less (K0) ubiquitin mutant. Unfortunately, as linear polyubiquitin is linked with head-to-tail linkage, both intact N-terminal and C-terminal of ubiquitin are required. A tag on either end of exogenous ubiquitin will not allow it to be incorporate properly into linear polyubiquitin chain. So there is a technical barrier for studying linear ubiquitination by expressing a tagged-ubiquitin K0 mutant in cells.

5. Figure 4B : there is no effect of the HOIP siRNA on TAK1 activation. Do the authors imply that linear polyubiquitination plays a different role in DNA damage- vs TNF-induced NF-kB activation ?

As we discussed in our response to comment #3 of reviewer 1, we do believe the functional significance of NEMO linear ubiquitination is relatively less critical in mediating TAK1 activation by a strong NF- B activator such as TNF , compared to that by genotoxic stress. This functional difference is consistent with the notion that NEMO is only required for genotoxic stress-induced TAK1 activation, but not for TAK1 activation by LPS or TNF as we reported previously (Wu et al. Mol Cell 2010).

6. Seeing an effect with the K285/309 mutation does not necessarily implicate a role for linear ubiquitination. K285 is also a site for monoubiquitination and K309 for sumoylation. This should be acknowledged in the Discussion.

We appreciated the reviewer's critical thinking and helpful suggestion. We have performed additional experiments to further support LUBAC's function in genotoxic NF- B signaling, which indicated the additive phenotypes observed from NEMO K285/309R mutant. (Please also see our response to comment (7) of reviewer 2). We have included addition discussion on this topic in our manuscript.

7. On page 18 the authors write : 'Then, the linear polyubiquitin chains on NEMO and the K63-polyubiquitin chains on ELKS may serve as platforms for recruiting TAK1 and IKK complexes' This is confusing : i) if NEMO recruits TAK1/TAB2 through linear chains, then ELKS is no longer required. However binding of TAB2 to linear chains is very weak. ii) If both IKK and TAK1 complexes are recruited to ELKS, then linear polyubiquitination is no longer necessary. iii) If IKK/NEMO is recruited to the K63 chains of ELKS, and TAK1 to the linear chains of NEMO, all molecules play a role, but again the affinity of TAB2 for linear chains is quite weak. In fact the only protein that seems to exhibit reasonable affinity for linear chains is NEMO itself. The authors should modify their discussion accordingly.

We thank the reviewer's insightful comments and, to further clarify the potential mechanisms, we have modified our discussion as following:
"NEMO was shown to bind to linear polyubiquitin with a higher affinity compared to K63-linked polyubiquitin, it is plausible that the NEMO-attached linear polyubiquitin may serve as binding platform for further recruitment of cytoplasmic NEMO-associated IKK complexes. Previous studies demonstrated that TAB2 preferentially binds to K63-linked polyubiquitin, and we found TAB2 may also interact with linear ubiquitin chain. Therefore, TAK1/TAB2 complex may primarily associate with the K63-polyubiquitin chains on ELKS, and with NEMO linear polyubiquitin to a lesser extent, upon genotoxic stress. The intertwined association could promote IKK /NEMO, TAK1/TAB2, K63-polyubiqutin and linear polyubiquitin to form a stabilized framework for optimal activation of both kinases, leading to efficient NF- B activation upon genotoxic stimulation"

8. The authors should also discuss how the different modifications occur: NEMO is first sumoylated on K277 and 309, then desumoylated and monoubiquitinated on the same Lys residues, and also monoubiquitinated on Lys 285. Then linear polyUb chains are added on Lys 285 and 309 : does it require deubiquitination of Lys 285, or extension of the monoUb by LUBAC ? The situation would be different for Lys 309 where desumoylation would be followed by linear polyubiquitination ?

In our revised Fig 3A, we now include data showing NEMO modification (Sumoylation, mono-ubiquitination and linear polyubiquitination) at different time point after genotoxic stimulation.

These findings strongly suggest a sequential order of NEMO Sumoylation, Mono- and linear polyubiquitination after genotoxic treatment. We have shown that K277/309 of NEMO were sumoylated then mono-ubiquitinated upon genotoxic stress, which are both nuclear events and should involve desumoylation process. Our fractionation data (Fig 3C) further suggested that NEMO linear ubiquitination took place in the cytoplasm. A recent publication (Hinz et al. Mol Cell, 2010) reported NEMO can be mono-ubiquitinated at K285 in cytoplasm in response to genotoxic stress. It is not entire clear whether the NEMO mono-ubiquitination at K277/309 or K285 are sequential or in parallel. However, by comparing the kinetics of K285 mono-ubiquitination (peaked around 60 min, Hinz et al.) and linear ubiquitination (Fig 3C), we believe NEMO K285 mono-ubiquitination takes place earlier than linear ubiquitination on the same residue by DNA damage. It is interesting to note that a recent report (Inn et al, Mol Cell. 2011, 41:354-65.) showed that mono-ubiquitination of TRIM25 makes it susceptible for LUBAC-dependent polyubiquitination. It is possible that mono-ubiquitination of NEMO on K285 facilitates LUBAC-dependent further linear polyubiquitination, by either promoting NEMO:LUBAC association or serving as a cornerstone for further extension of linear polyubiquitin. In either scenario, removing mono-ubiquitin from NEMO K285 will not be required. However, the underlying mechanisms still remain to be further investigated. We have added these points into the revised manuscript.

9. Minor point : Figure 2C : what is the intense band in the upper panel ? Immunoglobulins ? MW markers should be included.

The intense band is immunoglobulin heavy chain. We have repeated the experiment and improved results were shown in revised Fig 2D. MW marker has been included. We used a light chain-only secondary antibody in this set of experiments, so the heavy chain is no longer visible.

We thank all the reviewers for their critical reading and insightful comments to our manuscript, and we believe these comments and suggestions have helped us to greatly strengthen the studies presented in the current manuscript. We hope the reviewers find the quality of the data presented here improved and in support of our conclusions.

2nd Editorial Decision 09 May 2011

Thank you for your patience while the resubmission of your previously reviewed manuscript EMBOJ-2010-76307 was evaluated once more by the three original referees. As you will see from the enclosed reports, referee 3 is now satisfied, but referees 1 and 2 retain some major reservations that still preclude publication in The EMBO Journal at this point.

The overlapping main concern of both critical referees is that alternative scenarios to the invoked role of LUBAC-mediated NEMO linear polyubiquitination in the genotoxic stress response cannot be definitely ruled out. In particular, referee 1 (point 2) considers it possible that LUBAC may only be involved in a secondary NF-kB activation wave following initial ATM-dependent but LUBAC-independent NF-kB induction of TNF secretion. At the same time, both referees remain skeptical about the functional significance of NEMO linear ubiquitination (ref 1 point 1, ref 2 point 1): its occurrence would have to be more decisively demonstrated, and it would have to be ruled out that upstream effects in the pathway would be affected by NEMO K285/309 mutations, or that LUBAC inhibition could affect pathway activation in a NEMO-independent manner.

The other concerns of referees 1 and 2 represent mostly addressable specific technical and control issues (e.g. ref 1 points 6, ref 2 point 2), or aspects of discussion and presentation (ref 1 points 3, 4, 7); and one point is in my view well beyond the scope of this study (ref 1 point 8). Nevertheless, referee 1 raises one more substantial technical issue (point 5), regarding the discrepancy between HOIP levels in HOIL-1-deficient fibroblasts between your current work and a recent publication by some of you - this definitely also has to be clarified before eventual publication.

In conclusion, I am afraid that at this stage, we are still not in a position to strongly commit to

publication of your manuscript. We note that the paper would clearly become a much stronger candidate if the overlapping main concerns about indirect effects (ref 1 point 2, ref 2 point 1) could be decisively addressed and ruled out. I would thus like to give you the opportunity to address this main problem through an additional round of regular revision. However, I have to make it absolutely clear that the critical referees will have to become convinced by decisive additional data, otherwise we will see ourselves forced to ultimately reject the manuscript after this final round of re-revision. I realize that this may entail substantial further efforts, and that it may be difficult to obtain strong supporting evidence; I would therefore also understand if you decided to seek publication without significant changes elsewhere at this point (in which case I would ask you to kindly inform us briefly of such a withdrawal). Should you however feel confident that you might be able to obtain the requested substantiation, then please return a re-revised manuscript, including an exhaustive point-by-point response to all the reviewers' points, to us using the link below. In case you should have any further questions, please feel free to contact me.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Niu et al. now provide more convincing evidence for a role of endogenous LUBAC in genotoxic stress-induced IKK and TAK1 activation as well as stimulation-dependent interactions between endogenous LUBAC and NEMO. The quality of the Western Blots and the significance of the results have improved significantly. Additionally the authors extended their explanations in the result and discussion sections, putting their results in the context of previously published reports. However, the data regarding the role of linear ubiquitination of NEMO in genotoxic stress-induced NF-kB activation are still not convincing.

Major points:

1. I am still left with major concerns regarding Fig. 2 in which the authors claim that they show a LUBAC-mediated linear ubiquitination of NEMO on residues K285/309. Specifically on the basis of the result shown in Fig. 2B they cannot claim that the ubiquitin chains they detect are attached to NEMO because this pull-down was not performed under denaturing conditions or following a denaturation step. Also the effect exerted by etoposide treatment on the detected ubiquitin smear is at best marginal. In Fig. 2D a smear is already detected below the molecular weight of NEMO. How is that possible if the chains are attached to NEMO? Unspecific bands should be indicated (e.g. the prominent band at ~40 kDa).

2. In Fig. 3 data are shown in support of a role of LUBAC and linear ubiquitination of NEMO downstream of sumoylation, ATM-mediated phosphorylation and monoubiquitination of NEMO following genotoxic stress. Recently Biton et al. published that genotoxic stress triggers a first wave of ATM-dependent NF-kB activation which induces TNF and that this then triggers a second wave of NF-kB activation (Biton et al., Cell 2011). As LUBAC has been shown to play a role in TNF-induced NF-kB activation the question arises whether the role of LUBAC in genotoxic stress-induced NF-kB activation, proposed here to be a direct consequence of genotoxic stress, could be an indirect consequence of ATM-dependent TNF induction by genotoxic stress and therefore only a reflection of the role of LUBAC in TNF-induced NF-kB activation. This can be tested by inhibiting TNF and by testing whether, and if so, when TNF might be induced by genotoxic stress in the systems used here.

3. In Fig. 5 the authors claim that TAB2 binds to linearly ubiquitinated NEMO, though they do not show it. Despite an improvement of the experimental set-up, now using endogenous conditions, the results are still overinterpreted. It might well be possible that TAB2 does not directly interact with

linear ubiquitin chains attached to NEMO but that both proteins form part of a stimulation-induced multi-protein complex. Again, evidence for linear ubiquitination of NEMO as a consequence of etoposide treatment presented in this figure is weak and at best correlative.

4. Since the authors show an effect of genotoxic stress-induced TAK1 activation, their data presented in the answer letter to the reviewer regarding decreased phosphorylation of JNK should be shown in the supplementary information. Accordingly, on page 11 the authors should re-phrase their sentence since it is not "surprising" that TAK1 activity is also affected. They themselves claim that "NEMO is indispensable for genotoxic stress-induced TAK1 activation", making it likely that, if NEMO is affected by LUBAC knock-down, TAK1 activity would also be decreased. Including this Figure in the manuscript would further strengthen the point that LUBAC is involved in TAK1-induced JNK activation.

5. Recently Iwai, a coauthor on this study, published that HOIP levels are decreased in HOIL-1-/- MEFs (Nature, 471, page 635, Fig. 3c). Here the authors now claim that HOIP levels are unaffected in these cells. Which result is true? This is not trivial because consistency of published data is important and in addition downregulation of HOIP in the absence of HOIL-1 could also affect linear ubiquitination induced by a complex consisting of SHARPIN and HOIP (Ikeda et al., Nature 2011; Tokunaga et al., Nature 2011; Gerlach et al., Nature 2011). This issue has to be resolved.

6. On page 9 the authors claim that "we detected a significant increase of NEMO poly-ubiquitination upon etoposide treatment in cytosolic fractions but not in nuclear fractions". Since different amounts of NEMO are pulled down from the two fractions, they cannot be compared and therefore the above conclusion cannot be drawn. Equal amounts of NEMO pulled down from cytosolic versus nuclear fractions have to be compared.

7. In the discussion section on page 21 the authors mention that TNF-induced TAK1 activity might not be that prominently affected as TNF-induced rapid RIP1 ubiquitination. In this context only K63-linked ubiquitination of RIP1 is discussed. Recent papers showed that this concept is simplified since RIP1 is modified by additional ubiquitin chain linkages (Dynek et al., EMBO Journal 2011; Gerlach et al., Nature 2011). The discussion should reflect these recent findings.

8. As mentioned above, three groups, including one led by a coauthor of this study, Iwai, recently showed that LUBAC contains a further component known as SHARPIN. Though I do not expect the authors to perform all experiments regarding a possible influence of SHARPIN on genotoxic stress-induced signalling, given that there is overlap in authorship between the two studies, an analysis of SHARPIN in some of the key experiments should be included and the corresponding results should be discussed.

Referee #2 (Remarks to the Author):

This revised manuscript from Niu et al is a significant improvement from their earlier submission. Overall it is better controlled and the data are more convincing. However, there remain some additional concerns.

(1) While it seems clear that NEMO is linear ubiquitinated after DNA damage, its functional significance is not clear. This is for two reasons. The authors have not adequately controlled for NEMO independent effects of LUBAC inhibition, while the K285/309 mutant, used extensively, clearly has additional effects (for example, the authors on page 11 say this mutant 'only mildly affected' NEMO sumoylation, while to me, the effect seen in Fig. S2B seems very strong). The authors' model implies a sequential series of events, culminating in NEMO linear ubiquinatation in the cytoplasm. Therefore it is important that they demonstrate that HOIP depletion as well as expression of LUBAC-CS, do not affect these prior events. These include NEMO nuclear localisation, sumoylation and phosphorylation. Furthermore, it is very important that they show that LUBAC inhibition does not affect ATM activation. Finally, they need to show that LUBAC inhibition does not itself affect ELKS ubiquination.

(2) Although the authors have included a time course of NF-kB activation (Fig. S1D) this is with CPT. Yet the vast majority of experiments in the manuscript, including the functional analysis, are

with etoposide. As different types of DNA damage display differences in NF-kB activation, this experiment needs to be repeated with etoposide as the stimulus.

Referee #3 (Remarks to the Author):

The revised version of this manuscript answers my original criticisms and should now be suitable for publication in the EMBO Journal.

1st Revision - authors' response 22 June 2011

Thanks for considering our manuscript (EMBOJ-2011-77642) entitled "LUBAC Regulates NF- *k*B Activation upon Genotoxic Stress by Promoting Linear Ubiquitination of NEMO" for publication in the EMBO Journal. We have revised the manuscript in order to best address the comments of the reviewers. A point-by-point answer to each of the reviewer's comments is provided as follows.

Referee #1:

1. I am still left with major concerns regarding Fig. 2 in which the authors claim that they show a LUBAC-mediated linear ubiquitination of NEMO on residues K285/309. Specifically on the basis of the result shown in Fig. 2B they cannot claim that the ubiquitin chains they detect are attached to NEMO because this pull-down was not performed under denaturing conditions or following a denaturation step. Also the effect exerted by etoposide treatment on the detected ubiquitin smear is at best marginal. In Fig. 2D a smear is already detected below the molecular weight of NEMO. How is that possible if the chains are attached to NEMO? Unspecific bands should be indicated (e.g. the prominent band at ~40 kDa).

As we described in Methods section, to detect ubiquitinated or sumoylated form of NEMO, we lyzed cells with immunoprecipitation lysis buffer containing 1% SDS and incubated at 95∞C for 30 min. Then the lysates were diluted with IP lysis buffer (without SDS) to reduce SDS to 0.1% for NEMO immunoprecipitation. We consider our lysis condition is very stringent which should disrupt most, if not all, non-covalent modification and protein association of NEMO. This method was used in all the experiments for detecting NEMO ubiquitination/sumoylation throughout the manuscript, and has been used to study protein ubiquitination/sumoylation by others as well as in our previous publications. We are confident that the polyubiquitin signal detected in Fig2B represents NEMO ubiquitination. To address the reviewer's concern, we further repeated the experiment and improved data has been shown in revised Fig 2B. We also clarified the lysis condition in respective figure legend. In Fig 2D, we believe the smear/background bands below 72KDa marker are non-specific, since they were smaller than unmodified Myc6x-NEMO and appeared disregarding treatments. The prominent band below 43KDa marker represents immunoglobin light chain. We have added annotation in the figure legend as the reviewer suggested.

2. In Fig. 3 data are shown in support of a role of LUBAC and linear ubiquitination of NEMO downstream of sumoylation, ATM-mediated phosphorylation and monoubiquitination of NEMO following genotoxic stress. Recently Biton et al. published that genotoxic stress triggers a first wave of ATM-dependent NF-kB activation which induces TNF and that this then triggers a second wave of NF-kB activation (Biton et al., Cell 2011). As LUBAC has been shown to play a role in TNF-induced NF-kB activation the question arises whether the role of LUBAC in genotoxic stress-induced NF-kB activation, proposed here to be a direct consequence of genotoxic stress, could be an indirect consequence of ATM-dependent TNF induction by genotoxic stress and therefore only a reflection of the role of LUBAC in TNF-induced NF-kB activation. This can be tested by inhibiting TNF and by testing whether, and if so, when TNF might be induced by genotoxic stress in the systems used here.

To examining whether TNF plays a role in genotoxic NF- B signaling at early stage after genotoxic

stimulation, we quantified the TNF production/secretion in culture media of HEK293 cells after etoposide (10 μM) treatment. TNF concentration in culture media was not significantly increased until 8 hours after Etop treatment (s-Fig 3A), suggesting TNF may not play an important role in early responding NF- B signaling upon genotoxic treatment. Moreover, TNF induced at 20 h (~16 pg/ml) after 10 μM etoposide treatment, which we used throughout the manuscript, was much lower than that (~60-100 pg/ml) by 100 μM etoposide treatment used by Biton et al. Consistently, TNF neutralizing antibody, which effectively inhibited TNF-induced NF- B activation, did not significantly affect NF- B activation at 2 h after treatment with various genotoxic drugs (s-Fig 3B). Finally, Etop-induced NEMO linear ubiquitination can still be detected at 2 h after treatment in the presence of TNF neutralizing antibody(s-Fig 3C). All these evidence suggests that TNF may not be involved in NF- B activation at early stage after low level of genotoxic stimulation, which is consistent with the conclusion drawn by Biton et al.

3. In Fig. 5 the authors claim that TAB2 binds to linearly ubiquitinated NEMO, though they do not show it. Despite an improvement of the experimental set-up, now using endogenous conditions, the results are still overinterpreted. It might well be possible that TAB2 does not directly interact with linear ubiquitin chains attached to NEMO but that both proteins form part of a stimulation-induced multi-protein complex. Again, evidence for linear ubiquitination of NEMO as a consequence of etoposide treatment presented in this figure is weak and at best correlative.

We have rephrased the text by stating TAB2 may form a protein complex with linear ubiquitinated NEMO in cells upon genotoxic stimulation (Pg. 14).

4. Since the authors show an effect of genotoxic stress-induced TAK1 activation, their data presented in the answer letter to the reviewer regarding decreased phosphorylation of JNK should be shown in the supplementary information. Accordingly, on page 11 the authors should re-phrase their sentence since it is not "surprising" that TAK1 activity is also affected. They themselves claim that "NEMO is indispensable for genotoxic stress-induced TAK1 activation", making it likely that, if NEMO is affected by LUBAC knock-down, TAK1 activity would also be decreased. Including this Figure in the manuscript would further strengthen the point that LUBAC is involved in TAK1-induced JNK activation.

We have rephrased the text as the reviewer suggested (Pg.11). The JNK phosphorylation data are now included as supplementary Fig 4A.

5. Recently Iwai, a coauthor on this study, published that HOIP levels are decreased in HOIL-1-/- MEFs (Nature, 471, page 635, Fig. 3c). Here the authors now claim that HOIP levels are unaffected in these cells. Which result is true? This is not trivial because consistency of published data is important and in addition downregulation of HOIP in the absence of HOIL-1 could also affect linear ubiquitination induced by a complex consisting of SHARPIN and HOIP (Ikeda et al., Nature 2011; Tokunaga et al., Nature 2011; Gerlach et al., Nature 2011). This issue has to be resolved.

After discussion with our collaborator, we found that the HOIP antibody we used in our previous experiments was raised against human HOIP, which detect a non-specific signal at very similar size of HOIP in MEF cells. This antibody was used in Dr. Iwai's early publication (BBRC 2006, 351: 340-347, Fig 3E), where they showed similar signal in HOIL-1-deficient MEFs compared with wild type cells. They later raised a new antibody against mouse HOIP, which was used in their recent publications. By using this new mouse HOIP antibody, we now clearly show that HOIP level is significantly decreased in HOIL-1-/- and SHARPIN-/- MEFs (Fig 1E, Fig 4C-D, s-Fig 1D-F). Further we did observe stabilization of HOIP in HOIL-1-/- MEFs reconstituted with HA-HOIL (Fig 1D).

6. On page 9 the authors claim that "we detected a significant increase of NEMO poly-ubiquitination upon etoposide treatment in cytosolic fractions but not in nuclear fractions". Since different amounts of NEMO are pulled down from the two fractions, they cannot be compared and therefore the above conclusion cannot be drawn. Equal amounts of NEMO pulled down from cytosolic versus nuclear fractions have to be compared.

Based on our previous experiments and data shown in suppl-Fig 2C, we believe the cells with NEMO nuclear translocation or the amount of NEMO sequestered in nuclei upon DNA damage is

less than 5% of total cell or NEMO population. It is technically challenging to show similar amount of NEMO in both nuclear and cytoplasmic fraction upon DNA damage. We were not able to obtain similar amount of nuclear NEMO, compared with cytoplasmic NEMO, by using 5 times more cells for nuclear fraction than that for cytoplasmic fraction, even though we found cytoplasmic contamination in nuclear fraction was increased due to the much larger number of cells used. Our fractionation data also indicated that HOIP predominantly resides in cytoplasm (s-Fig 2C), which further supported our observation of NEMO linear ubiquitination in cytoplasm. To avoid inaccuracy, we have revised the text by stating "we detected a significant increase of NEMO polyubiquitination upon etoposide treatment in cytosolic fractions."(Pg.10)

7. In the discussion section on page 21 the authors mention that TNF-induced TAK1 activity might not be that prominently affected as TNF-induced rapid RIP1 ubiquitination. In this context only K63-linked ubiquitination of RIP1 is discussed. Recent papers showed that this concept is simplified since RIP1 is modified by additional ubiquitin chain linkages (Dynek et al., EMBO Journal 2011; Gerlach et al., Nature 2011). The discussion should reflect these recent findings.

Following the reviewer's suggestion, we have modified the discussion section to reflect the recent progress of RIP1 ubiquitination in TNF signaling pathway. (Pg. 22)

8. As mentioned above, three groups, including one led by a coauthor of this study, Iwai, recently showed that LUBAC contains a further component known as SHARPIN. Though I do not expect the authors to perform all experiments regarding a possible influence of SHARPIN on genotoxic stress-induced signalling, given that there is overlap in authorship between the two studies, an analysis of SHARPIN in some of the key experiments should be included and the corresponding results should be discussed.

We obtained SHARPIN deficient (cpdm) MEFs from Dr. Iwai and examined whether genotoxic NF-B signaling is impaired in these MEFs. As shown in Fig 1E, we found DNA damage-induced NF- B activation is almost abolished in SHARPIN-/- MEFs, supporting a critical role of the trimeric LUBAC complex in genotoxic NF- B signaling.

Referee #2:

(1) While it seems clear that NEMO is linear ubiquitinated after DNA damage, its functional significance is not clear. This is for two reasons. The authors have not adequately controlled for NEMO independent effects of LUBAC inhibition, while the K285/309 mutant, used extensively, clearly has additional effects (for example, the authors on page 11 say this mutant 'only mildly affected' NEMO sumoylation, while to me, the effect seen in Fig. S2B seems very strong). The authors' model implies a sequential series of events, culminating in NEMO linear ubiquinatation in the cytoplasm. Therefore it is important that they demonstrate that HOIP depletion as well as expression of LUBAC-CS, do not affect these prior events. These include NEMO nuclear localisation, sumoylation and phosphorylation. Furthermore, it is very important that they show that LUBAC inhibition does not affect ATM activation. Finally, they need to show that LUBAC inhibition does not itself affect ELKS ubiquitination.

To address the reviewer's concern, we depleted HOIP in HEK293 cells with siRNA and examined Etoposide treatment-induced ATM activation, NEMO sumoylation/phosphorylation/ nuclear translocation, as well as ELKS ubiquitination. As shown in Suppl-Fig 2A-D, HOIP knockdown did not overtly inhibit any of these events examined, suggesting LUBAC may contribute to genotoxic NF- B signaling by primarily promoting NEMO linear ubiquitination. The decrease of sumoylation of NEMO K285/309R mutant is likely due to the mutation on K309 which is required for NEMO sumoylation. We have modified the text by stating "NEMO sumoylation was still induced by genotoxic stimulation in cells expressing NEMO K285/309R mutant." (Pg. 12)

(2) Although the authors have included a time course of NF-kB activation (Fig. S1D) this is with CPT. Yet the vast majority of experiments in the manuscript, including the functional analysis, are with etoposide. As different types of DNA damage display differences in NF-kB activation, this experiment needs to be repeated with etoposide as the stimulus.

We now included data from additional time course experiments in HOIL-1 MEFs treated with Dox and etoposide (Suppl-Fig 1E-F), which are consistent with the data from CPT-treated cells.

Referee #3:

The revised version of this manuscript answers my original criticisms and should now be suitable for publication in the EMBO Journal.

We thank the reviewer's positive comments.

Acceptance letter 08 July 2011

Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the original reviewers, and it has also been seen once more by one of the original referees. I am happy to inform you that there are no further objections towards publication in The EMBO Journal!

Yours sincerely,

Editor
The EMBO Journal

