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E3 ubiquitin ligase NEDD4L negatively regulates inflammation by promoting ubiquitination of MEKK2

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Dear Dr. Lin

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also point out several technical concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed.

From the referee comments it is clear that a significant revision will be required to substantiate the findings. Yet, given the constructive comments, we would like to give you the chance to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (June 15, 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

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We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.

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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) Figure legends and data quantification:
The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
See also the guidelines for figure legend preparation:
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10) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready and please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

In this manuscript, Lin and co-workers report a molecular mechanism by which the inflammatory signaling triggered by extracellular cytokines are tightly regulated. They identified a HECT-type E3 ligase NEDD4L as a negative regulator of IL-17/TNFalpha/IL1alpha signaling. They found that NEDD4L interacts with MEKK2 and promotes its ubiquitination through K27-linked ubiquitin chains. NEDD4L inhibits IL17-induced inflammation in MEKK2-dependent manner. They also found that phosphorylation of MEKK2 induces MEKK2-dependent degradation to shutoff the signaling. Finally, using Nedd4l deficient mice, they showed that NEDD4L down-regulates inflammatory signaling in vivo. Taken together, they identified NEDD4L as a negative regulator of inflammation through promoting degradation of MEKK2.

Overall, the authors provide clear evidence that NEDD4L is a negative regulator of the inflammatory signaling triggered by IL-17/TNFalpha/IL1alpha and that NEDD4L promotes ubiquitination and degradation of MEKK2. Together with analyses using Nedd4l deficient mice, physiological significance of NEDD4L in inflammatory signalings is convincing. My concerns relate to the biochemical analysis of the ubiquitin code generated by NEDD4L: the authors claim that NEDD4L mediates K27-linked ubiquitination based on in vitro assays using ubiquitin mutants. However, drawing a conclusion based on such mutational experiments carries a risk of artifacts (see points 1 and 2). As the enzymatic property of NEDD4L is not an essential part of the authors' main finding, I suggest the authors reconsider this part of conclusion.

1) It is well established that the ubiquitin chain specificity of the HECT-type E3 ligases is determined by their C-terminal amino acids. NEDD4 family ligases, including NEDD4L, exhibit strict specificity toward K63 linkages (e.g., Maspero et al., Nat SMB 2013). The authors' conclusion (Fig. 4H and 4I) contradicts the well-established notion among the ubiquitin biology community and seems unlikely from the biochemical point of view.

2) The authors need to perform the mass spectrometry-based quantitative analysis of ubiquitin linkages to conclude that NEDD4L generates K27-linked ubiquitin chains. There is a lot of literature on the quantification of ubiquitin chains (e.g., Gygi and Harper; Peng; Komander), but the abundance of K27 linkages is very few in cells.

3) In Fig. 1F, the effects of NEDD4L deficiency in primary lung epithelial cells are not so impressive, compared with those in other cells (Fig. 1A to G). Please explain.

4) Fig. 3G: This is a critical experiment showing that NEDD4L affects turn-over of MEKK2. However, the blotting data of anti-MEKK2 is not of high quality. Moreover, the steady-state level of MEKK2 is higher in Nedd4l-/- cells (lanes 1 and 5), hampering assessment of turn-over of MEKK2 after the CHX addition. The authors should quantify band intensities from independent experiments.

5) Fig. 4D: Why the authors use MG115 in addition to MG132? In general, single use of MG132 (or bortezomib, epoxomicin, etc.) is sufficient to inhibit proteasomal degradation. Also, as K63-linked ubiquitin chains may promote lysosomal degradation, the authors should check the effect of bafilomycin A for the protein abundance of NEDD4L.

6) Please indicate the molecular weight markers for the blots of ubiquitination assays.

Referee #2:

In this manuscript the hypothesis is tested that NEDD4-2 inhibits inflammation by promoting ubiquitination of MEKK2. This is tested in transfected HeLa cells as well as in cells derived from Nedd4l (NEDD4-2) deficient mice. The essays involve IL-7 stimulation following various signalling in these cells. Most of the data demonstrate that under NEDD4-2 deficiency IL-17 induced pathways are stimulated. It is also shown that NEDD4-2 and MEKK2 constitutively interact with each other, and that NEDD4-2 negatively regulates stability of MEKK2. Nedd4l mice are more susceptible to IL-17-induced inflammation and symptoms of experimental autoimmune encephalomyelitis (EAE) are aggravated. This is an interesting study, however, a few points have to be addressed
1) A number of RNA interfering experiments, directed against NEDD4-2 or MEKK2, either in HeLa cells or MEFs are presented. The validation is not satisfactory. Only anti NEDD4-2 or anti MEKK2 WBs are provided. At least for one experiment the authors should demonstrate for each RNAi by co-transfection of a resistant NEDD4-2 or MEKK2 construct that the interference was specific and that the effect of NEDD4-2 or MEKK2 can be reconstituted.

2) Many Western blots are provided without quantification, for example in Fig. 3. WB of either HeLa (interference against Nedd4L or not) or MEFS, stimulated with IL-17, are shown to demonstrate increase in phosphorylation of p65, or p38, but these blots are not very convincing, therefore is required (Fig. 3A and B). Similarly, in Fig. 3E, the authors aim to demonstrate that overexpression of NEDD4L (but not its inactive mutant NEDD4L-CA) affect MEKK2 expression, but this is not obvious to the reader. Moreover, it is claimed that IL-17 induced phosphorylation of p-P65 or p-P38, but also this is not obvious to the reviewer. Quantification of the blots and statistics has to be provided for most of the figures.

3) Several experiments in Fig. 4, demonstrating MEKK2 ubiquitination, are not convincing. In Fig. 4B it looks as the same experiment is shown twice? An in vitro ubiquitination assay is presented in Fig. 4G, demonstrating that the Nedd4L C2 domain is required for MEKK2 ubiquitination. The data also suggest that Nedd4L without the catalytic HECT domain still promotes ubiquitination. Ho do the authors explain that finding?

4) Fig. 2. The authors should demonstrate that NEDD4-2 is expressed in peritoneal mesothelial cells.

5) When ubiquitination is studied after immunoprecipitation of a protein, co-precipitated, associated proteins may also be ubiquitinated. It should be attempted to carry out these immunoprecipitations under denaturing conditions. Alternatively, gels should include the regions below 100 kDa to assure that we are not looking at smaller associated proteins that are ubiquitinated.

6) Fig. 5. Also in these experiments the effect of interfering MEKK2 on p-P65 or p-P38 should be quantified. Indeed, just based on the immunoblots, the reduction of the phosphorylation is not obvious.

7) How was the antibody against MEKK Ser520 validated?

To Reviewer 1:

Thanks very much for your comments and suggestions regarding our manuscript entitled “E3 ubiquitin ligase NEDD4L negatively regulates inflammation by promoting K27-linked ubiquitination of MEKK2” (Manuscript ID EMBOR-2022-54603V1). According to your suggestions, we have added some new data and re-organized the manuscript. If you have any further questions, please inform us and we can discuss them further.

1.Question: *It is well established that the ubiquitin chain specificity of the HECT-type E3 ligases is determined by their C-terminal amino acids. NEDD4 family ligases, including NEDD4L, exhibit strict specificity toward K63 linkages (e.g., Maspero et al., Nat SMB 2013). The authors' conclusion (Fig. 4H and 4I) contradicts the well-established notion among the ubiquitin biology community and seems unlikely from the biochemical point of view.*

Response: Thanks for your suggestion. As you mentioned that NEDD4 family ligases, including NEDD4L, exhibit strict specificity toward K63 linkages. However, there are many papers which have reported that NEDD4 and NEDD4L can mediate substrate proteins ubiquitination in a non-K63 linkage manner, such as in a K29 linkage manner (Farooq *et al*, 2022; Gao *et al*, 2021),and also in a K27 linkage manner(Li *et al*, 2022; Liu *et al*, 2021; Pei *et al*, 2017; Xie *et al*, 2020; Xie *et al*, 2022). Thus, I think it is possible that NEDD4L mediates K27-linked ubiquitination of MEKK2 in our study.

According to your suggestion, I have re-placed the statements of“K27-linked ubiquitination of MEKK2” with “ubiquitination of MEKK2” in the conclusion part, which were marked in red, and also renamed the title as“E3 ubiquitin ligase NEDD4L negatively regulates inflammation by promoting ubiquitination of MEKK2”in revised manuscript .

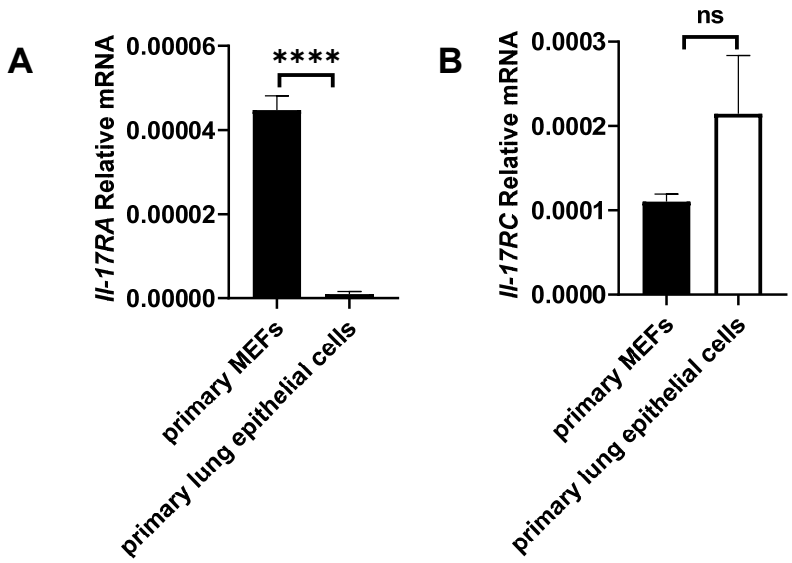
2.Question: *The authors need to perform the mass spectrometry-based quantitative analysis of ubiquitin linkages to conclude that NEDD4L generates K27-linked ubiquitin chains. There is a lot of literature on the quantification of ubiquitin chains*

(e.g., Gygi and Harper; Peng; Komander), but the abundance of K27 linkages is very few in cells.

Response: Thanks for your kind suggestions. It's true that abundance of K27 linkages is very few in cells, but it's an important modification of ubiquitination, which has been reported to play crucial roles in signaling cascades. As the abundance of K27 linkages is very few in cells, which may explain why the commercial antibody specific against to K27-linkage ubiquitin is still unavailable, while the commercial antibodies specific against to total ubiquitin, K48-linkage, and K63-linkage ubiquitin are already available and widely used in many papers. Thus, it's difficult to measure the endogenous K27-linkage ubiquitination of substrates. We have intended to perform the unbiased analysis, such as mass spectrometry-based quantitative analysis of ubiquitin linkages, to conclude that NEDD4L generates K27-linked ubiquitin chains on MEKK2. Unfortunately, the commercial spectrometry-based quantitative analysis of ubiquitin is still unavailable in our country. Mapping of exogenous wild-type Ub or mutant Ubs, including K6R/O, K11R/O, K27R/O, K29R/O, K33R/O, K48R/O, K63R/O, together with or without E3 and its potential substrates has been used to identify the possible subtype modification of ubiquitination. In order to confirm that NEDD4L generates K27-linked ubiquitin chains on MEKK2，we co-transfected Flag-MEKK2 and HA-Ub/HA-Ub-K27O/HA-Ub-K63O with or without Myc-NEDD4L in a dosage increasement dependent manner in HEK293T cells, and subsequently performed ubiquitination assay. As shown in **Figure 4J** in revised manuscript, the wild-type Ub-linked and K27O-linked ubiquitination of Flag-MEKK2 was significantly enhanced with the increasement of Myc-NEDD4L. However, the K63O-linked ubiquitination of Flag-MEKK2 remained constant, which may indicate that NEDD4L generates K27-linked ubiquitin chains on MEKK2 in a dosage dependent manner, but not a K63-linked dependent manner.

3.Question: *In Fig. 1F, the effects of NEDD4L deficiency in primary lung epithelial cells are not so impressive, compared with those in other cells (Fig. 1A to G). Please explain.*

Response: Thanks for your careful reading. We have noticed that the effects of NEDD4L deficiency in primary lung epithelial cells are not so impressive, compared with those in other cells. For IL-17R signaling was initialed by the binding of IL-17A/F with its receptors, IL-17RA or IL-17RC. To answer this question, we measure the *Il-17RA* and *Il-17RC* gene expression in primary lung epithelial cells and MEFs. As shown in **Response Fig1**, primary lung epithelial cells express relative lower gene expression of *Il-17RA* than that in primary MEFs, while the gene expression of *Il-17RC* is comparable. Thus, IL-17-induced signaling and genes expression are not so impressive in lung epithelial cells, compared with those in primary MEFs.



Response Figure1. *Il-17RA* gene expression in primary cells.

(A, B) Realtime RT-PCR analysis of *Il-17RA* **(A)** or *Il-17RC* **(B)** mRNA expression in primary lung epithelial cells and primary MEFs cells isolated from *Nedd4l*$^{+/+}$ mice. Data information: Data are shown as the mean ± s.e.m. and are representative of three independent experiments. Significant differences were tested using a two-tailed Student's t-test (E, F, G). NS, means no significance, ****P<0.00001.

4.Question: *Fig. 3G: This is a critical experiment showing that NEDD4L affects turn-over of MEKK2. However, the blotting data of anti-MEKK2 is not of high quality.*

Moreover, the steady-state level of MEKK2 is higher in Nedd4l-/- cells (lanes 1 and 5), hampering assessment of turn-over of MEKK2 after the CHX addition. The authors should quantify band intensities from independent experiments.

Response: Thanks for your suggestion. We replace the **Figure3G** with a blotting data of better-quality in the revised manuscript, and also quantify band intensities from independent 3 experiments. The data showed that NEDD4L deficiency in MEFs cells significantly prolongs the half-live of MEKK2 protein with the treatment of CHX **(Figure3H)**.

5.Question: *Fig. 4D: Why the authors use MG115 in addition to MG132? In general, single use of MG132 (or bortezomib, epoxomicin, etc.) is sufficient to inhibit proteasomal degradation. Also, as K63-linked ubiquitin chains may promote lysosomal degradation, the authors should check the effect of bafilomycin A for the protein abundance of NEDD4L.*

Response: MG115 is potent, reversible 20S and 26S proteasome inhibitor, and MG132 is targeted 26S proteasome complex. Single using of MG132 is sufficient to inhibit proteasomal degradation. Combination of these two inhibitors can completely inhibit proteasomal degradation. According to your suggestion, we repeated the data as indicated in **Figure 4D** in revised manuscript. The MEFs were treated by DMSO, MG132, or bafilomycin A (Baf A1) to check in which pathway NEDD4L mediates MEKK2 degradation. As shown in **Figure 4D**, NEDD4L mediates MEKK2 degradation in a proteasome-dependent pathway but not in a lysosomal-dependent pathway.

6.Question: *Please indicate the molecular weight markers for the blots of ubiquitination assays.*

Response: According to your suggestion, we have added the molecular weight markers for the blots of ubiquitination assays in revised manuscript.

To Reviewer 2:

Thanks very much for your comments and suggestions regarding our manuscript entitled “E3 ubiquitin ligase NEDD4L negatively regulates inflammation by promoting K27-linked ubiquitination of MEKK2” (Manuscript ID EMBOR-2022-54603V1). According to your suggestions, we have added some new data and re-organized the manuscript. If you have any further questions, please inform us and we can discuss them further.

1.Question: *A number of RNA interfering experiments, directed against NEDD4-2 or MEKK2, either in HeLa cells or MEFs are presented. The validation is not satisfactory. Only anti NEDD4-2 or anti MEKK2 WBs are provided. At least for one experiment the authors should demonstrate for each RNAi by co-transfection of a resistant NEDD4-2 or MEKK2 construct that the interference was specific and that the effect of NEDD4-2 or MEKK2 can be reconstituted.*

Response: Thanks for your suggestion. We have performed the reconstitution assays of NEDD4L/NEDD4L-C942A or MEKK2/MEKK2-S520A in *Nedd4l* or *Mekk2* silenced HeLa cells, respectively. As shown in **Figure EV3E and Figure EV4H** in revised manuscript, the effect of NEDD4L or MEKK2 can be reconstituted, which indicated that the RNAi interference was of good specificity.

2.Question: *Many Western blots are provided without quantification, for example in Fig. 3. WB of either HeLa (interference against Nedd4L or not) or MEFS, stimulated with IL-17, are shown to demonstrate increase in phosphorylation of p65, or p38, but these blots are not very convincing, therefore is required (Fig. 3A and B). Similarly, in Fig. 3E, the authors aim to demonstrate that overexpression of NEDD4L (but not its inactive mutant NEDD4L-CA) affect MEKK2 expression, but this is not obvious to the reader. Moreover, it is claimed that IL-17 induced phosphorylation of p-P65 or p-P38,*

but also this is not obvious to the reviewer. Quantification of the blots and statistics has to be provided for most of the figures.

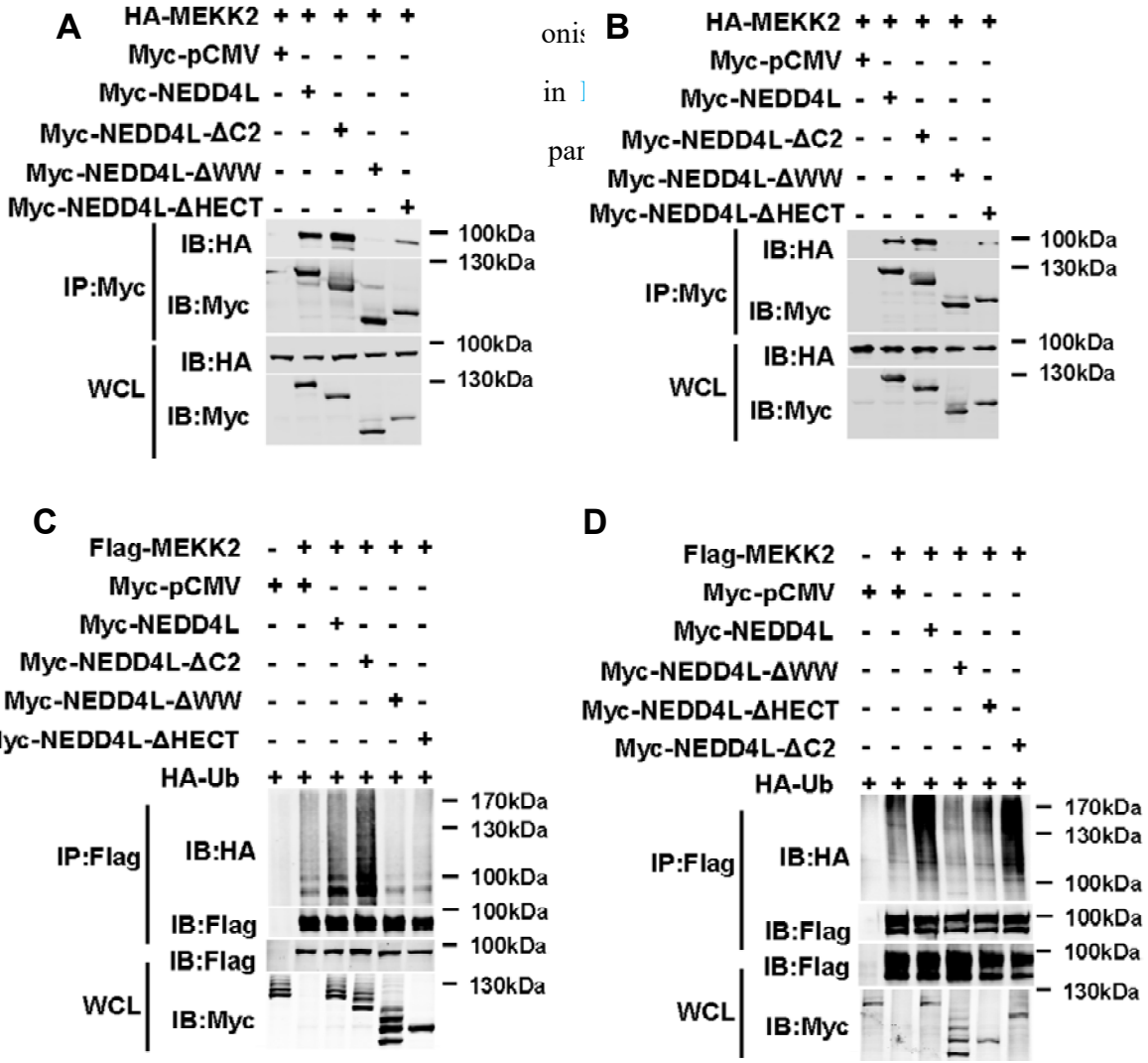
Response: According to your suggestion, the intensity of phosphorylated proteins normalized to total protein or MEKK2 to actin has been calculated using the Image J software. And the numbers have been added under the phosphorylated proteins and MEKK2 blots in revised manuscript.

3.Question: *Several experiments in Fig. 4, demonstrating MEKK2 ubiquitination, are not convincing. In Fig. 4B it looks as the same experiment is shown twice? An in vitro ubiquitination assay is presented in Fig. 4G, demonstrating that the Nedd4L C2 domain is required for MEKK2 ubiquitination. The data also suggest that Nedd4L without the catalytic HECT domain still promotes ubiquitination. How do the authors explain that finding?*

Response: Sorry for the confuse. **In Figure 4B**, the *in vitro* GST-tagged pulldown assay was carried out in a reaction mixture containing GST-null, GST-MEKK2, GST-beads, and NEDD4L proteins. The input proteins were from 1/10 pulldown assays mixture. That is why Figure 4B looks as the same experiment was shown twice, even though the blots were from two membranes.

Sorry for the terrible mistake. It has been well-established that the WW domain of NEDD4 family ligases play critical role for recognizing and binding the substrate proteins. However, our previous data showed that NEDD4L interact with MEKK2 dependent on C2 domain of NEDD4L which contradicted the well-established notion. So, we repeated the mapping experiments of interaction and also ubiquitination carefully. As shown in **Figure 4C and 4G** in revised manuscript, deletion of WW domain, but not C2 and HECT domain, disrupted the interaction and ubiquitination between NEDD4L and MEKK2, demonstrating that the WW domain was necessary for NEDD4L to bind and ubiquitinate to MEKK2. NEDD4L without the catalytic HECT domain largely impaired the capability of NEDD4L to promote MEKK2 ubiquitination. However, deletion of C2 domain promoted the ubiquitination of MEKK2, which may be resulted from the relieve of autoinhibitory interaction

between its C2 and HECT domains, consistently with the notion that C2 domain of HECT family ligases, including smurf2,NEDD4, and NEDD4L, inhibit the function onis in] par



Response Figure2. Similar repeated experiments related to Figure 4C and 4G.

(A, B) Immunoblot of HA-MEKK2 co-immunoprecipitated with Myc-tag specific antibody from lysates of HEK293T cells co-transfected with plasmids expressing HA-MEKK2 and Myc-tagged wild type NEDD4L or mutant NEDD4L with C2 (ΔC2, WW (ΔWW) or HECT (ΔHECT) domain deleted.

(C, D) Immunoblot analysis of total ubiquitination of Flag-MEKK2 following

precipitation of MEKK2 with Flag-tag specific antibody from lysates of HEK293T cells co-transfected with plasmids expressing Flag-MEKK2 and Myc-tagged wild type NEDD4L or mutant NEDD4L with C2 (ΔC2, WW (ΔWW) or HECT (ΔHECT) domain deleted.

4.Question: *Fig. 2. The authors should demonstrate that NEDD4-2 is expressed in peritoneal mesothelial cells.*

Response: According to your suggestion, we have added NEDD4L expression blot in peritoneal mesothelial cells, which is shown in **Figure EV2E** of revised manuscript.

5.Question: *When ubiquitination is studied after immunoprecipitation of a protein, co-precipitated, associated proteins may also be ubiquitinated. It should be attempted to carry out these immunoprecipitations under denaturing conditions. Alternatively, gels should include the regions below 100 kDa to assure that we are not looking at smaller associated proteins that are ubiquitinated.*

Response: Thanks for your kind suggestion. All the ubiquitination assays were done as described (Liu *et al.*, 2021; Zhong *et al*, 2012). For ubiquitination assay, immunoprecipitates were re-extracted in lysis buffer containing 1% SDS and denatured by heating for 10 mins. Supernatants were diluted with lysis buffer until the concentration of SDS was decreased to 0.1%, followed by re-immunoprecipitation with the appropriate antibodies. Thus, there would be no other associated ubiquitinated proteins detected in our assays.

6.Question: *Fig. 5. Also in these experiments the effect of interfering MEKK2 on p-P65 or p-P38 should be quantified. Indeed, just based on the immunoblots, the reduction of the phosphorylation is not obvious.*

Response: According to your suggestion, the intensity of phosphorylated proteins normalized to total protein has been calculated using the Image J software. And the numbers have been added under the phosphorylated proteins blots in revised manuscript.

7.Question: *How was the antibody against MEKK Ser520 validated?*

Response: We constructed the MEKK2 Ser520 continuously activated mutant plasmids (Flag-MEKK2 S520D, Flag-MEKK2 S520E), and MEKK2 inactivated mutant plasmid (Flag-MEKK2 S520A). After transfection of plasmids into HEK293T cells, western blotting was performed and the antibody against MEKK Ser520 was used to detect the specific bands. As shown in **Figure EV4F** in revised manuscript, the bands of Flag-MEKK2 S520D and Flag-MEKKS520E showed strong phosphorylation intensity, the band of wild-type Flag-MEKK2 showed medium phosphorylation intensity, however, the band of Flag-MEKK2 S520A showed lowest phosphorylation intensity compared to that of other MEKK2 mutants. Besides, we also immunoprecipitated the Flag-MEKK2 protein from HEK293T cells, and then incubated the protein with calf intestinal alkaline phosphatase (CIAP) for 30 mins. After incubation, the phosphorylation band of MEKK2 was vanished **(Figure EV4G)**, suggesting that the bands detected by the antibody against MEKK Ser520 were specific.

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The authors have made the demanded changes and replied to all my concerns.

The authors have addressed all editorial requests.

Dr. Wenlong Lin
Institute of Immunology and The Second Affiliated Hospital
School of medcine
Yuhangtang Road 866
hangzhou, Zhejiang 310058
China

Dear Dr. Lin,

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The data shown in figures should satisfy the following conditions:

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