EMBO reports

NEDD4L-mediated Merlin ubiquitination facilitates Hippo pathway activation

Yiju Wei, Patricia Yee, Zhijun Liu, Lei Zhang, Hui Guo, Haiyan Zheng, Benjamin Anderson, Melissa Gulley and Wei Li
DOI: 10.15252/embr.202050642

Corresponding author(s): Wei Li (weili@pennstatehealth.psu.edu)

Review Timeline:

Submission Date:	14th Apr 20
Editorial Decision:	19th May 20
Revision Received:	17th Aug 20
Editorial Decision:	15th Sep 20
Revision Received:	17th Sep 20
Accepted:	18th Sep 20

Editor: Achim Breiling

Transaction Report:

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

1st Editorial Decision 19th May 2020

Dear Dr. Li,

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think that the findings are of interest, but they also have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication in EMBO reports. As the reports are below, and I think all points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your 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 of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs

to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

For more details please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation

See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) 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: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

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

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

NEDD4L-mediated Merlin ubiquitination facilitates Hippo pathway activation in growth control.

The authors show new data that the E3 ubiquitin ligase NEDD4L is responsible for merlin ubiquitination important for promoting Hippo pathway regulation (via Lats1 binding and activation). Moreover they identified an activation cascade where dephosphoylation of S518 and subsequent conformational changes allows the accessibility of merlin to E3 ubiquitin ligase. This dephosphorylation and ubiquitination cascade generates an ubiquitin-mediated merlin scaffold for Lats1-leading to Hippo pathway activation. Taken together the authors describe an interesting mechanism of merlin in proliferation control that contributes to its function as a tumor suppressor.

In principle I am positive about the manuscript it is mostely well written and concise. The presentation of the in vitro and in vivo results are good and their conclusions appear to well

founded and supported by the data. I have some comments below.

Comments

1. To separate references in text something other than a comma (,) should be used, ie semicolon (;). Referring to the comma after the dates. Otherwise it is difficult to discern where one finishes and another starts. Page 2 and throughout the manuscript.
2. Traditionally only the first author is needed when referring to paper and the authors are more than two. Page 2 and throughout the text.
3. "In the purified ubiquitinated proteins, endogenous Merlin detected by anti-Merlin antibody was more abundant in cells treated with thapsigargin than with DMSO control (Figure 1G, upper panel)." Are the authors referring to total ubiquitination or some specific WB bands? Is this generic ubiquitination or some specific bands of the ubiquitination? We would agree with the upregulation of poly-ubiquitinated Merlin (the bands above the arrow) but with such exposure of the Merlin band it is hard to judge if there is upregulation of a mono-ubiquitinated Merlin (whichever band/species the arrow corresponds to). The authors need to provide either a clearer blot, or a more specific text. Page 5 and relevant Figure 1.
4. "As seen in probing of total lysates, enhanced mono- and dual-ubiquitination was observed by this ubiquitination detecting assay when cells were detached (Figure 1H, arrowheads and arrows, respectively)".
Maybe a longer exposure for the dual ubiquitination should be provided. It can be placed in the supplementary information. Page 5 and relevant Figure 1.
5. Why is there a shift in the MW of TAZ? Is it due to the phosphorylation of TAZ? If yes, probe with p-TAZ. Page 5 and relevant Figure 1.
6. Fig. 1E, Why phospo-Taz hasn't been used here? Fig. 1E
7. "This approach confirmed that Merlin ubiquitination can be induced by NEDD4L (Figure 3G, comparing lanes 3 and 2)."
In Fig. 3F and G AMOTL1 tagged with either Myc (F) or HA (G) increases on it's own Merlin's ubiquitination (lanes 1 and 2). Why is that? is it because it recruits the endogenous NEDD4L? If this is the case AMOTL1 expression with NEDD4L knock-down should abolish this increased ubiquitination. Page 7 and Fig. 3F and G.
8. "In contrast, the 2PY*+LY* AMOTL1 mutant has no such ability (Figure 4F, comparing lanes 2 and 3), suggesting that interaction between AMOTL1 and NEDD4L is important for promoting Merlin ubiquitination." If reading this right, Fig. 4F lanes 2, 3 and 4 show the total Merlin along with the mono- and poly-ubiquitinated Merlin. However, the mutant 2PY* seems to reduce the poly-ubiquitination but keeps the enhanced mono-ubiquitination of Merlin but the 2PY*+2LY* mutant reduced even the mono-ubiquitination of Merlin. Any explanation on this? Page 8 and Fig. 4F.
9. ¬Materials and methods, section Mice. No mouse tissue lysate has been used on the manuscript. Why tissues lysates are being described in the methods? Page 14.
10. How many mice were used per timepoint in the tumorigenesis experiment? Page 14.
11. Graph Fig 2F, what do the data points represent? Biological repetitions or counted cells? Page 25 and Fig 2F.
12. Graph Fig. 7D, the counted dots per cell, do they represent one biological experiment?
13. General points for the whole manuscript.
• In the figures the molecular weight is abbreviated as Kd. It should be kDa.
• In all the graphs, the data points should represent the average of each biological experiment. The N numbers should be reported on all graphs, along with the actual P values for significant and non-significant values.
• All experiments need to be repeated at least 3 times to have any meaningful statistical testing and validity.

• The authors should submit to the journal all the WBs with each panel being repeated at least 3 times in independent experiments.

Referee #2:

In this report, the authors study the regulation of the tumour suppressor protein Merlin/NF2 by posttranslational modifications. Merlin function is known to be regulated by phosphorylation, which is thought to promote conformational changes in the structure of Merlin. Wei et al. explore the possibility that other posttranslational modifications are involved in the regulation of Merlin function and identify that Merlin is ubiquitinated. The authors propose that ubiquitination of Merlin is required for its function and is promoted by dephosphorylation of Ser 518. Ubiquitination of Merlin is mediated by NEDD4L and requires AMOT1. Wei et al. define Lys 396 as the major ubiquitination site in Merlin. Mutation of the putative ubiquitination site or depletion of NEDD4L impairs Merlin ubiquitination and Merlin binding to Lats1, leading to inhibition of Lats1 activation.

This is a highly intriguing report on the role of NEDD4L in the regulation of Hippo signaling and likely to spur debate in the field given the fact that NEDD4L has been previously identified as a regulator of both AMOT1 and Lats and to promote the degradation, rather than the activation, of these Hippo pathway proteins.

Overall, this manuscript has interesting new observations related to the regulation of Merlin/NF2, one of the Hippo pathway genes for which there is extensive genetic evidence of its role in cancer due to the frequent mutations found in neurofibromatosis type 2 and associated sporadic tumors. However, there are particular issues with the manuscript that prevent its publication in its current form. Below are some points to be addressed by the authors.

Major points:

My main criticism of the manuscript relates to the fact that the authors seem to ignore several reports containing data that seem to contradict the results they have obtained. Previous results have shown several instances where NEDD4 family proteins (NEDD4, NEDD4L, ITCH, etc.) have been shown to promote degradation of Hippo pathway components (Lats, AMOT, etc.). Also, the authors fail to reference previous reports that could potentially explain some of their observations, such as the fact that mono-ubiquitination of AMOT proteins promotes Lats activation. There should be, at the very least, extensive discussion of the authors' results in the context of previous findings and, if possible, additional experimental validation to address the fact these results are contradictory to published data. It may be necessary to address whether the mechanism discovered by the authors is (or not) active in the cell line models where NEDD4 family members have been shown to lead to degradation of AMOT or Lats.

Where possible, quantification of specific results should be performed. For instance, the levels of ubiquitinated Merlin are sometimes challenging to assess as the proportion of Merlin that is modified is low and it is difficult to determine if the overall levels of Merlin are consistent between samples due to the massively exposed main band for Merlin. It is possible that some of the differences seen are due to different total levels of Merlin and, in fact, the relative levels of ubiquitinated Merlin are not significantly altered.

Related to Figures 1 and S1:

Why are there high levels of Merlin ubiquitination in panel 1A? Do the cells used normally have high basal levels of Hippo pathway activity (since the authors later find that Merlin ubiquitination is associated with Lats1 activation)? Were the protein lysates obtained in the presence of deubiquitinating enzyme inhibitors?
Were other modes of activation of Hippo signaling besides cell detachment and Ca2+ signaling tested? Do the authors see the same effect when cells are contact inhibited or in high density?
If possible, authors should show in Figure S1B controls corresponding to attached cells and not just samples obtained in the context of cell detachment.

Related to Figure 2:
Do the authors have any results regarding regulation of LATS1 or YAP phosphorylation when the expression levels of PAK, PKA or MYPT-PP1 are modulated?
Regarding panel 2H, do the authors have similar results when experiments were performed with His-tagged ubiquitin?

Related to Figure 3 and S3:
Do the authors have mass spectrometry data from cells not treated with thapsigargin? Is the interaction between NEDD4L and Merlin induced by thapsigargin treatment or do they interact in basal conditions as well?
Authors found that depletion of DCAF1 or DDB1 did not affect Merlin ubiquitination in the context of thapsigargin treatment. Do they have similar results in the cell detachment context (or others)?
Are there other NEDD4 family proteins able to ubiquitinate Merlin or is this a NEDD4L-specific function? Previous reports have shown that AMOT proteins can be targeted by several NEDD4 family members (e.g. NEDD4, NEDD4L, ITCH; Wang et al. Biochem J 2012) so it is formally possible that this is also the case for Merlin.
Is HUWE1 also involved in Merlin ubiquitination?

Related to Figure 4 and S4:
Authors should discuss previous data suggesting that AMOT proteins are ubiquitinated and degraded by NEDD4 family proteins (Wang et al. Biochem J 2012; Skouloudaki and Walz, PLoS One 2012) and how this relates to the regulation of Merlin. Did the authors validate these results in the cell lines studied, i.e., are AMOT proteins degraded in the presence of NEDD4L? Could this indicate that Merlin ubiquitination is the result of a "bystander" effect of AMOT degradation? The authors clearly see AMOTL1 ubiquitination in Figure 3F.
If indeed AMOTL1 is required for mediating the interaction between Merlin and NEDD4L, is this function compensated for by other AMOT proteins, since this seems to be dependent on the PPXY and LPXY motifs, which are conserved in (at least) AMOT/p130?

Related to Figure 5 and S5:
Data from panels 5F, 5G and 5H suggests that K396 is not the only residue that is modified when Merlin is ubiquitinated. Do the authors have similar results with the double Lysine mutant? Also, is there a reason why there are so many nonspecific bands in panel 5F when previous Ni2+ assays had none?

Related to Figure 6 and S6:
Do the authors have any data from Merlin KO cells reconstituted with S518A mutant, other Lysine mutants or a Merlin mutant that cannot interact with AMOTL1? The latter would be important to give strength to the argument that AMOTL1 is necessary for NEDD4L to regulate Merlin function.
Authors suggest that NEDD4L is required for Lats1 activation. How can this be reconciled with previous results suggesting that NEDD4 is responsible for Lats degradation (Bae et al. Nat

Commun 2015)?

Related to Figure 7:
PLA images corresponding to panel 7G should be provided in supplementary information.
Authors suggest that mutation of Lysine 396 specifically affects binding of Merlin to Lats1, but not to other interacting proteins. Is the binding between Merlin and Lats1 dependent on the UBA domain of Lats? It has been previously shown that AMOTL2 mono-ubiquitination activates Lats1/2 (Kim et al. EMBO Rep 2015). Given that the authors have seen AMOTL1 ubiquitination (Figure 3F), which is consistent with mono-ubiquitination, is it possible that the activation of Lats1 is in fact dependent on AMOTL1 ubiquitination?

Related to Figure 8 and S8:
Does NEDD4L modulate Hippo signaling in Meso-33 cells in the absence of ectopic expression of Merlin? If so, is this AMOTL1-dependent? Authors have only assessed BrdU and not provided any specific Hippo readouts.

Minor points:
In Figure S1B, why did authors only assess TAZ and not YAP?

Figure S8 should be Figure S7.

Referee #3:

Summary:
1-The manuscript reports a key finding: Merlin ubiquitination at K396 is central to the activation of Lats1 kinase and subsequently of the Hippo signaling pathway.
2-The finding is potentially of significance as it describes a new mechanism for NF2/Hippo activity regulation.
3- The work will be of interest to a large audience as the Hippo pathway is involved in a wide range of cellular functions and diseases.
4- The major finding is robustly documented.

Comments:

The manuscript by Wei and colleagues uncovers a new regulation mechanism of the activity of the NF2/Merlin tumor suppressor. The study clearly shows that Merlin is ubiquitinated on lysine 396 by the ubiquitine ligase NEDD4L. Access of NEDD4L to Merlin is provided by the scaffold AmotL1. Merlin ubiquitination facilitates the interaction with the kinase Lats1 for Hippo pathway activation. This prevents Yap/Taz nuclear localization and thus cell proliferation and tumor formation in nude mice xenograft model. In conclusion, the study claims that Merlin ubiquitination is a necessary step for Merlin activation of the Hippo pathway and, consequently, the inhibition of cellular growth.
The study is well organized and of good quality. The experimental design is sound. The results are convincing and will be of interest for the community of scientists working on NF2 and Hippo signaling.
This interesting work would, in my opinion, deserve publication in Embo report at the condition that the authors address three important questions in greater details as well a few minor points:

Major Points:
I-How relevant is the proposed mechanism for growth control?

a) Merlin ubiquitination is observed here under selected growth conditions (Calcium signaling and loss of adhesion) in a set of cell lines. Given that the most striking cellular phenotype associated to NF2 inactivation is the loss of contact inhibition of growth, could the author also investigate Merlin ubiquitination in this context? For example (but these are suggestions only), the authors could:

- Provide kinetics of Merlin ubiquitination upon increasing cell density over 48 hours.
- They could compare Merlin WT and K396R impact on cell growth and saturation density.

b) From the figures, it appears that only a very small pool of Merlin is ubiquitinated. This is in strong contrast to the phosphorylation of Merlin on S518 that usually concerns most Merlin molecules. Only combined overexpression of NEDD4L and AmotL1 leads to a more robust signal of the ubiquitinated Merlin species (Figure 4F). However, the impact of Merlin ubiquitination on Lats1 binding and activation is very strong (Figure 6B, D and 7C). We would then expect Lats1 to be in complex preferentially with ubiquitinated Merlin.

- Quantification of the percentage of monoubiquitinated Merlin vs unmodified Merlin should be provided in several figures (1A, C, D, 2G etc… everywhere a conclusion is derived from an observed change in Merlin ubiquitination.
- Proximity Ligation Assays are an acceptable way to show proximity but interaction should be validated by additional techniques like co-immunoprecipitation of Merlin and Lats1 .
- More importantly, the author should evaluate the ratio of ubiquitinated vs unmodified Merlin bound to Lats1. The relative abundance of the two Merlin species should be measurable by western-blot following coIP.

c) In addition to AmotL1, Amot and AmotL2 also bind to Merlin. Besides NEDD4L, the motin family was shown to interact with NEDD4 and Itch of the NEDD4 family (Wang et al Biochem. J. (2012) 444, 279-289). Are the observations presented in the manuscript relevant only for AmotL1 and NEDD4L?

- Could the authors test as proof-of-principle the effect of Amot, AmotL2, NEDD4 or Itch on Merlin ubiquitination? An experimental approach similar to Figure 3F would be sufficient.
- Furthermore, the endogenous levels of AmotL1 need to be presented when siRNA or shRNA are used (Figure 4A and 4B).
- Likewise, the endogenous levels of NEDD4L and AMOTL1 need to be documented in Figure 1A.

d) Merlin was previously shown to trigger Amot degradation via NEDD4. In the context of the manuscript, this could represent a feedback mechanism tto limit Merlin/Lats1 activation.
- Could the author evaluate the impact of Merlin WT, 518D and K396R expression on AmotL1 levels(e.g. with the cells used to generate Figure 8 H-K)?

II-How specific on Lats1 interaction is Merlin ubiquitination?

Lats1 binds to the FERM domain of Merlin like several other interactors. The authors could test how specific is the impact of ubiquitination on FERM domain interactors. For example:
- How do EBP50, alpha-tubulin or CD44 bind to Merlin K396R compared to Merlin WT or S518A upon cotransfection with NEDD4L and AmotL1.
- How does the C-terminal domain of Merlin co-IP with Merlin K396R compared to Merlin WT or S518A upon cotransfection with NEDD4L and AmotL1.
These experiments could help to discriminate between a specific regulation of Lats1 binding or a

more global change in Merlin conformation.

III- How does this new mechanism fit with already known regulation of Merlin activity?

From the literature, it appears that a compact conformation of Merlin opens upon binding to PIP2 (Chinthalapudi et al 2018 Nat. Comm.). Phosphorylation of S518 doesn't appear to modify Merlin conformation but to inhibit the interaction with AmotL1 (Li et al 2015 cell research). Hence, as the authors discussed, Merlin ubiquitination appears as a step of a chain of events allowing Lats1 activation and cell growth inhibition.

However, to better convey the general message of the manuscript, it would be useful to provide a scheme that put the main finding of the study (i.e. Merlin ubiquitination and impact on Lats/Hippo) into the context of what is already known about the mechanisms of Merlin regulation (e.g. in Figure 4C).

Minor points:

1.) Subcellular localization of Merlin K396R is never shown. IF and /or cell fractionation should be presented in comparison to S518A (membrane enriched) and S518D (cytosol enriched). One cannot exclude that K396R mutation alters subcellular localization. Also, using fractionation, it would be possible to evaluate the distribution of ubiquitinated Merlin and refine the model.

2.) There is no indication of the expression level of Merlin WT, 518D or K396R in the cells used in the sphere assay and the mouse tumor assay. This is necessary.

3.) The duration of cell suspension is not mentioned and should be clarified in the Material and Methods section. Does ubiquitination increase with time? Does it parallel with Lats1 phosphorylation?

4.) The tumor sphere assay is not really detailed. I guess the authors are using an agarose layer to prevent adhesion?

5.) The paper from K. Chinthalapudi et al. (2018. Lipid binding promotes the open conformation and tumor-suppressive activity of neurofibromin 2. Nat. Comm.) relative to merlin activation by PiP2 binding should be cited. As well as Li et al 2015. Angiomotin binding-induced activation of Merlin/NF2 in the Hippo pathway. Cell research) showing the opening and activation of Merlin via the binding to motins.

Conclusion:
The study has the potential to be very interesting to a broad readership. However, in my opinion, it needs to address a couple important complementary questions in order to strengthen the relevance of the findings. Not all the suggested experiments need to be performed necessarily but the main points should be addressed.

REVIEWER COMMENTS

Referee #1:

NEDD4L-mediated Merlin ubiquitination facilitates Hippo pathway activation in growth control.

The authors show new data that the E3 ubiquitin ligase NEDD4L is responsible for merlin ubiquitination important for promoting Hippo pathway regulation (via Lats1 binding and activation). Moreover they identified an activation cascade where dephosphoylation of S518 and subsequent conformational changes allows the accessibility of merlin to E3 ubiquitin ligase. This dephosphorylation and ubiquitination cascade generates an ubiquitin-mediated merlin scaffold for Lats1-leading to Hippo pathway activation. Taken together the authors describe an interesting mechanism of merlin in proliferation control that contributes to its function as a tumor suppressor.

In principle I am positive about the manuscript it is mostly well written and concise. The presentation of the in vitro and in vivo results are good and their conclusions appear to well founded and supported by the data. I have some comments below.

Comments

1. To separate references in text something other than a comma (,) should be used, ie semicolon (;). Referring to the comma after the dates. Otherwise it is difficult to discern where one finishes and another starts. Page 2 and throughout the manuscript.

We have fixed this issue by using semicolons.

2. Traditionally only the first author is needed when referring to paper and the authors are more than two. Page 2 and throughout the text.

We have fixed this issue.

3. "In the purified ubiquitinated proteins, endogenous Merlin detected by anti-Merlin antibody was more abundant in cells treated with thapsigargin than with DMSO control (Figure 1G, upper panel)."
Are the authors referring to total ubiquitination or some specific WB bands? Is this generic ubiquitination or some specific bands of the ubiquitination? We would agree with the upregulation of poly-ubiquitinated Merlin (the bands above the arrow) but with such exposure of the Merlin band it is hard to judge if there is upregulation of a mono-ubiquitinated Merlin (whichever band/species the arrow corresponds to). The authors need to provide either a clearer blot, or a more specific text. Page 5 and relevant Figure 1.

We have clarified this by adding more specific text stating “The difference is clearer for di- or tri-ubiquitinated Merlin than for mono-ubiquitinated Merlin (Figure 1G, arrow).” on page 5.

4. "As seen in probing of total lysates, enhanced mono- and dual-ubiquitination was observed by this ubiquitination detecting assay when cells were detached (Figure 1H, arrowheads and arrows, respectively)".
Maybe a longer exposure for the dual ubiquitination should be provided. It can be placed in the supplementary information. Page 5 and relevant Figure 1.

We have provided longer exposure of Merlin in lysate and precipitate to show the dual ubiquitination more clearly in Figure EV1D.

5. Why is there a shift in the MW of TAZ? Is it due to the phosphorylation of TAZ? If yes, probe with p-TAZ. Page 5 and relevant Figure 1.

The shift in the molecular weight of TAZ is usually caused by the phosphorylation of TAZ. We have probed with p-TAZ to show the increase of TAZ phosphorylation in Figure 1E.

6. Fig. 1E, Why phospo-Taz hasn't been used here? Fig. 1E

We have probed with phospho-TAZ and added this to Figure 1E and page 4.

7. "This approach confirmed that Merlin ubiquitination can be induced by NEDD4L (Figure 3G, comparing lanes 3 and 2)."
In Fig. 3F and G AMOTL1 tagged with either Myc (F) or HA (G) increases on it's own Merlin's ubiquitination (lanes 1 and 2). Why is that? is it because it recruits the endogenous NEDD4L? If this is the case AMOTL1 expression with NEDD4L knock-down should abolish this increased ubiquitination. Page 7 and Fig. 3F and G.

AMOTL1 expression increases on its own Merlin's ubiquitination is presumably because it recruits the endogenous NEDD4L. Following the reviewer's suggestion, we tested this by knocking down NEDD4L. As expected, Merlin ubiquitination was reduced upon NEDD4L knockdown in cells with and without recombinant AMOTL1. AMOTL1 expression can still slightly increase the ubiquitination in NEDD4L-silenced cells (Figure EV2F). This effect may be due to incomplete NEDD4L depletion or other E3 ligases recruited by overexpressed AMOTL1. A description of this result has been added on page 7.

8. "In contrast, the 2PY*+LY* AMOTL1 mutant has no such ability (Figure 4F, comparing lanes 2 and 3), suggesting that interaction between AMOTL1 and NEDD4L is important for promoting Merlin ubiquitination." If reading this right, Fig. 4F lanes 2, 3 and 4 show the total Merlin along with the mono- and poly-ubiquitinated Merlin. However, the mutant 2PY* seems to reduce the poly-ubiquitination but keeps the enhanced mono-ubiquitination of Merlin but the 2PY*+2LY* mutant reduced even the mono-ubiquitination of Merlin. Any explanation on this? Page 8 and Fig. 4F.

The mutant 2PY* slightly reduces the poly-ubiquitination and mono-ubiquitination of Merlin compared to wild-type AMOTL1. This is likely due to the slightly reduced binding between AMOTL1 (2PY*) and NEDD4L, because NEDD4L co-immunoprecipitated by AMOTL1(2PY*) appears to be slightly less than that by AMOTL1 (WT) (comparing lanes 4 to 2). For AMOTL1(2PY*+LY*), because it has no detectable interaction with NEDD4L, it abolished all of the effects on Merlin ubiquitination.

9. ¬Materials and methods, section Mice. No mouse tissue lysate has been used on the manuscript. Why tissues lysates are being described in the methods? Page 14.

We have deleted this part in Materials and Methods.

10. How many mice were used per timepoint in the tumorigenesis experiment? Page 14.

We have provided the mice numbers (n_{Vector}=8, n_{WT}=10, n_{K396R}=10, n_{S518D}=8.) in Figure 8J legend on page 36.

11. Graph Fig 2F, what do the data points represent? Biological repetitions or counted cells? Page 25 and Fig 2F.

The data points in Figure 2F (Figure EV1F in the revised version) represent individual cells that were quantified. We have added this information in the figure legend on page 36.

12. Graph Fig. 7D, the counted dots per cell, do they represent one biological experiment?

Each data point in Figure 7D represents an image field containing an average of 10 cells. All images were collected from one experiment. Two independent experiments were performed and they showed similar results. We have added the information in the figure legend on page 35.

13. General points for the whole manuscript.
• In the figures the molecular weight is abbreviated as Kd. It should be kDa.

We have changed to kDa.

• In all the graphs, the data points should represent the average of each biological experiment. The N numbers should be reported on all graphs, along with the actual P values for significant and non-significant values.

We have defined the data points and provided the N numbers of all graphs in the figure legends. P values, when significant, we have classified based on cut-offs commonly used in statistics; when non-significant, we have provided the actual values in the figures.

• All experiments need to be repeated at least 3 times to have any meaningful statistical testing and validity.

For most of the experiments in the graphs, we have shown the results from 3 or more biological repeats. For the PLA experiments and YAP/TAZ localization experiments, the quantification was focused on individual cells. In each experiment, we randomly examined 40-200 (PLA) and 16-17 cells (YAP/TAZ localization). We have performed two independent experiments in each case, and they showed similar results. We have provided the information in each figure legend.

• The authors should submit to the journal all the WBs with each panel being repeated at least 3 times in independent experiments.

We have repeated at least 2 times for most of the experiments and more times for the key results. These experiment results were intermingled with others during running of the western gels. Because of the large numbers of western blotting results in the manuscript, we respectively suggest that preparing for submission of all of the repeated WBs results might be unrealistic if not undoable in a reasonable time frame. To alleviate the reviewer's concern, we have provided the unprocessed/uncropped blots for each WB shown in the figures.

Referee #2:

In this report, the authors study the regulation of the tumour suppressor protein Merlin/NF2 by posttranslational modifications. Merlin function is known to be regulated by phosphorylation, which is thought to promote conformational changes in the structure of Merlin. Wei et al. explore the possibility that other posttranslational modifications are involved in the regulation of Merlin function and identify that Merlin is ubiquitinated. The authors propose that ubiquitination of Merlin is required for its function and is promoted by dephosphorylation of Ser 518. Ubiquitination of Merlin is mediated by NEDD4L and requires AMOT1. Wei et al. define Lys 396 as the major ubiquitination site in Merlin. Mutation of the putative ubiquitination site or depletion of NEDD4L impairs Merlin ubiquitination and Merlin binding to Lats1, leading to inhibition of Lats1 activation.

This is a highly intriguing report on the role of NEDD4L in the regulation of Hippo signaling and likely to spur debate in the field given the fact that NEDD4L has been previously identified as a regulator of both AMOT1 and Lats and to promote the degradation, rather than the activation, of these Hippo pathway proteins.

Overall, this manuscript has interesting new observations related to the regulation of Merlin/NF2, one of the Hippo pathway genes for which there is extensive genetic evidence of its role in cancer due to the frequent mutations found in neurofibromatosis type 2 and associated sporadic tumors. However, there are particular issues with the manuscript that prevent its publication in its current form. Below are some points to be addressed by the authors.

Major points:

My main criticism of the manuscript relates to the fact that the authors seem to ignore several reports containing data that seem to contradict the results they have obtained. Previous results have shown several instances where NEDD4 family proteins (NEDD4, NEDD4L, ITCH, etc.) have been shown to promote degradation of Hippo pathway components (Lats, AMOT, etc.). Also, the authors fail to reference previous reports that could potentially explain some of their observations, such as the fact that mono-ubiquitination of AMOT proteins promotes Lats activation. There should be, at the very least, extensive discussion of the authors' results in the context of previous findings and, if possible, additional experimental validation to address the fact these results are contradictory to published data. It may be necessary to address whether the mechanism discovered by the authors is (or not) active in the cell line models where NEDD4 family members have been shown to lead to degradation of AMOT or Lats.

We have addressed these issues through addressing the following specific points raised by this reviewer.

Where possible, quantification of specific results should be performed. For instance, the levels of ubiquitinated Merlin are sometimes challenging to assess as the proportion of

Merlin that is modified is low and it is difficult to determine if the overall levels of Merlin are consistent between samples due to the massively exposed main band for Merlin. It is possible that some of the differences seen are due to different total levels of Merlin and, in fact, the relative levels of ubiquitinated Merlin are not significantly altered.

We have quantified the ratio of mono-ubiquitinated Merlin to the main Merlin band in each related western blot. The ratio is now shown under each blot panel.

Related to Figures 1 and S1:
Why are there high levels of Merlin ubiquitination in panel 1A? Do the cells used normally have high basal levels of Hippo pathway activity (since the authors later find that Merlin ubiquitination is associated with Lats1 activation)? Were the protein lysates obtained in the presence of deubiquitinating enzyme inhibitors?

The cells used in Figure 1A were cultured in regular conditions at a steady state. When the protein lysates were collected, there were no deubiquitinating enzyme inhibitors added. The results showed various levels of Merlin ubiquitination in these cells. We have probed the Hippo pathway components and added the results in Figure EV1A to show the Hippo pathway activity. In such conditions, the phosphorylation of Hippo pathway effectors, such as Lats1, YAP and TAZ, did not appear to fully correlate with Merlin ubiquitination. This is probably because the culture condition is at a steady state. We have added the information on page 4.

Were other modes of activation of Hippo signaling besides cell detachment and Ca2+ signaling tested? Do the authors see the same effect when cells are contact inhibited or in high density?

We have examined Merlin ubiquitination in the context of contact inhibition. However, we did not see a clear trend of change in the ubiquitination (Figure R1)[Figures for referees not shown.] . These results suggested that the mode of Merlin regulation described in this manuscript may not simply apply to contact inhibition. Notably, contact inhibition appears to be a slow and progressively developed phenomenon, which usually occurs in several days. In contrast, cell detachment and Ca^{2+} signaling tested in the manuscript usually occur within 30 minutes to several hours. Such a time scale difference suggested that the mode of Merlin regulation may be more prominent or more readily detectable when the regulatory process occurs

relatively quickly. We acknowledge that determination of whether similar regulation is involved in other modes of Hippo pathway activation is interesting and have added this perspective into the discussion on page 17.

If possible, authors should show in Figure S1B controls corresponding to attached cells and not just samples obtained in the context of cell detachment.

We have added the results showing attached cells as well in Figure S1B (Figure EV1C in the revised version).

Related to Figure 2:
Do the authors have any results regarding regulation of LATS1 or YAP phosphorylation when the expression levels of PAK, PKA or MYPT-PP1 are modulated?
Regarding panel 2H, do the authors have similar results when experiments were performed with His-tagged ubiquitin?

We have examined the effect when active PAK is expressed. The result demonstrated that promoting Merlin phosphorylation by stably expressing an active PAK mutant T423E in LN229 cells showed similar inhibitory effects on thapsigargin-induced Merlin ubiquitination and Lats1 phosphorylation (Figure 2E). This result is consistent with our observation when Rac is activated (Figure 2D), and it therefore further supports that Merlin dephosphorylation promotes its ubiquitination and activation. This result has been described on page 6.

Regarding panel 2H (2G in the revised manuscript), we have used two alternative approaches to examine the ubiquitination. These included accessing the higher molecular weight Merlin form (Figure 2F) and a common ubiquitination-detection assay based on probing ubiquitin followed by immunoprecipitation (Figure 2G). Both approaches showed similar results. Therefore, we did not further test using the third approach with His-tagged ubiquitin.

Related to Figure 3 and S3:
Do the authors have mass spectrometry data from cells not treated with thapsigargin? Is the interaction between NEDD4L and Merlin induced by thapsigargin treatment or do they interact in basal conditions as well?

We have conducted mass spectrometry from cells not treated with thapsigargin and included the data in Figure 3A. After normalizing against Merlin, we found that NEDD4L was more enriched in the product co-immunoprecipitated with Merlin upon thapsigargin treatment (Figure EV2A). This result suggested that the interaction between NEDD4L and Merlin is enhanced by thapsigargin treatment, although they show some interaction in basal conditions as well. This notion was further confirmed by the co-immunoprecipitation assay (Figure EV2D). A description of this result has been added on page 7.

Authors found that depletion of DCAF1 or DDB1 did not affect Merlin ubiquitination in the context of thapsigargin treatment. Do they have similar results in the cell detachment context (or others)?

We have conducted the experiment in the cell detachment context and found that depletion of DCAF1 or DDB1 did not inhibit Merlin ubiquitination (Figure EV2C). Therefore, they have similar results to the context of thapsigargin treatment. A description of this result has been added on page 6.

Are there other NEDD4 family proteins able to ubiquitinate Merlin or is this a NEDD4L-specific function? Previous reports have shown that AMOT proteins can be targeted by several NEDD4 family members (e.g. NEDD4, NEDD4L, ITCH; Wang et al. Biochem J 2012) so it is formally possible that this is also the case for Merlin.
Is HUWE1 also involved in Merlin ubiquitination?

We have examined whether NEDD4 and ITCH are able to ubiquitinate Merlin. The results showed that these proteins do have the ability to ubiquitinate Merlin when overexpressed (Figure EV2G and EV2H). These suggested that inducing Merlin ubiquitination may not be a NEDD4L-specific function. A description of these results has been added on page 8.

Based on the mass spectrometry result, the interaction between HUWE1 and Merlin appears to be much weaker than that between NEDD4L and Merlin (Figure 3A). In addition, unlike NEDD4L, whose interaction with Merlin is enhanced by thapsigargin treatment (Figure 3A and EV2A), the interaction between HUWE1 and Merlin appears to be even weaker upon thapsigargin treatment than upon treatment with DMSO (Figure 3A and EV2A). Therefore, HUWE1's role in this case, if any, might be much less significant than NEDD4L. With this, we respectively suggest that investigation of HUWE1 may be out of the scope of this study.

Related to Figure 4 and S4:
Authors should discuss previous data suggesting that AMOT proteins are ubiquitinated and degraded by NEDD4 family proteins (Wang et al. Biochem J 2012; Skouloudaki and Walz, PLoS One 2012) and how this relates to the regulation of Merlin. Did the authors validate these results in the cell lines studied, i.e., are AMOT proteins degraded in the presence of NEDD4L? Could this indicate that Merlin ubiquitination is the result of a "bystander" effect of AMOT degradation? The authors clearly see AMOTL1 ubiquitination in Figure 3F.

We have validated the reported results. In our experiments, overexpression of NEDD4L with the AMOT family proteins in 293T cells showed that ubiquitination of AMOTL1 and AMOTL2 can be markedly induced. Such ubiquitination was accompanied by decrease of their expression (Figure EV3E). This result is consistent with previous reports. Although both AMOT proteins and Merlin can be ubiquitinated by NEDD4L, it may not necessarily mean that Merlin ubiquitination is a "bystander" effect of AMOT degradation. It is possible that although the AMOT family proteins are able to recruit

NEDD4L to activate Merlin, such recruitment may trigger its own ubiquitination and degradation. This may constitute a negative feedback loop to avoid uncontrolled Merlin activation. We agree with the reviewer that parallel ubiquitination of AMOT proteins and Merlin by NEDD4L is an intriguing phenomenon, and it warrants further studies in the future. A description of the new result was added on page 10. The discussion has been added on page 16.

If indeed AMOTL1 is required for mediating the interaction between Merlin and NEDD4L, is this function compensated for by other AMOT proteins, since this seems to be dependent on the PPXY and LPXY motifs, which are conserved in (at least) AMOT/p130?

We have examined whether AMOT and AMOTL2 are able to mediate Merlin ubiquitination. Overexpression of each protein with NEDD4L was able to induce Merlin ubiquitination similarly to AMOTL1 (Figure EV3D), suggesting that the AMOT family proteins may have similar properties in this setting. A description of these results has been added on pages 9 and 10.

Related to Figure 5 and S5:
Data from panels 5F, 5G and 5H suggests that K396 is not the only residue that is modified when Merlin is ubiquitinated. Do the authors have similar results with the double Lysine mutant? Also, is there a reason why there are so many nonspecific bands in panel 5F when previous Ni2+ assays had none?

We agree with the reviewer that, based on the data, K396 is not the only residue that is modified when Merlin is ubiquitinated. We are sorry for the unclear labeling. In Figure 5F, we examined the double lysine mutant (K159R+K396R, denoted by 159,396KR) and found that the double mutation did not further reduce ubiquitination. Therefore, we have focused on characterizing K396R since then, because K396 appeared to be the major ubiquitin conjugation site.

The nonspecific bands in panel 5F were presumably caused by the anti-HA antibody, whereas in previous Ni^{2+} assays (e.g. Figures 1G, 1H, 3E, 4B), an anti-Merlin antibody was used. The difference is presumably caused by distinct specificities of each antibody. Although the same anti-HA antibody was used in Figure EV3B, the assay used, the cell line, and expression level of the HA-tagged proteins were different. These differences may also cause the presence of these non-specific bands only in Figure 5F.

Related to Figure 6 and S6:
Do the authors have any data from Merlin KO cells reconstituted with S518A mutant, other Lysine mutants or a Merlin mutant that cannot interact with AMOTL1? The latter would be important to give strength to the argument that AMOTL1 is necessary for NEDD4L to regulate Merlin function.

Following the reviewer's suggestion, we have screened six NF2 patient-derived Merlin mutations for their interaction with AMOTL1 in Merlin KO cells. We found Δ513-521,

L517P, and L535P mutations markedly disrupted Merlin's binding to AMOTL1 (Figure EV3C). We then examined ubiquitination of these Merlin mutants. L535P and Δ513-521 markedly inhibited thapsigargin-induced Merlin ubiquitination, while L517P partially reduced such ubiquitination (Figure 4F). We used L535P to further examine Merlin ubiquitination induced by the AMTOL1-NEDD4L apparatus in the reconstituted system expressing these recombinant proteins. Although ubiquitination of wild-type Merlin can be robustly induced by AMOTL1 and NEDD4L, this effect was suppressed by Merlin L535P mutation (Figure 4G). A description of these results has been added on page 9. We further tested the function of these mutants in Hippo pathway activation using the Merlin KO cells reconstituted by Δ513-521, L517P, or L535P mutants. Thapsigargin-induced Lats1 and TAZ phosphorylation was compromised in these cells in comparison to wild-type Merlin-reconstituted cells (Figure 6C). A description of these results has been added on page 11. Overall, these results further supported that AMOTL1-binding is necessary for NEDD4L-induced Merlin ubiquitination and function in the Hippo pathway.

Authors suggest that NEDD4L is required for Lats1 activation. How can this be reconciled with previous results suggesting that NEDD4 is responsible for Lats degradation (Bae et al. Nat Commun 2015)?

We found that NEDD4 and Itch can also induce Merlin ubiquitination when overexpressed with AMOTL1 and Merlin. Although this result suggested that these two ligases may have similar functions to NEDD4L in this case, it needs to be cautiously interpreted because our initial mass spectrometry result did not find these proteins in the precipitates co-purified with Merlin (Figure 3A). Of note, although Itch and NEDD4 have been found to inactivate Lats kinases by promoting their degradation (Bae et al., 2015; Ho et al., 2011; Salah et al., 2013; Salah et al., 2011), NEDD4L was not reported to have such functions. The contrasting effects on Lats by NEDD4L versus Itch and NEDD4 suggested that different members in this E3 ligase family may regulate the Hippo pathway in opposite manners. We have added this discussion on page 16.

Related to Figure 7:
PLA images corresponding to panel 7G should be provided in supplementary information. Authors suggest that mutation of Lysine 396 specifically affects binding of Merlin to Lats1, but not to other interacting proteins. Is the binding between Merlin and Lats1 dependent on the UBA domain of Lats? It has been previously shown that AMOTL2 mono-ubiquitination activates Lats1/2 (Kim et al. EMBO Rep 2015). Given that the authors have seen AMOTL1 ubiquitination (Figure 3F), which is consistent with mono-ubiquitination, is it possible that the activation of Lats1 is in fact dependent on AMOTL1 ubiquitination?

We have provided the PLA images corresponding to panel 7G in Figure EV4E.

To examine whether the UBA domain is involved in binding to the conjugated ubiquitin on Merlin, we constructed a UBA domain deletion (ΔUBA) Lats1 mutant and examined its interaction with Merlin through co-immunoprecipitation. In DMSO-treated cells, the

Lats1 ΔUBA mutant showed a weaker ability than wild-type Lats1 in binding to the ubiquitinated Merlin (Figure EV4F, arrowhead, comparing lane 3 to 2). However, in thapsigargin-treated cells, the ΔUBA mutant bound to the ubiquitinated Merlin similarly to wild-type Lats1 (Figure EV4F, arrowhead, comparing lane 6 to 5). This result suggested that the Lats1 UBA domain is unnecessary for Merlin's binding to Lats1, at least when Merlin ubiquitination is induced by thapsigargin. A description of these results has been added on page 13.

Our results indicated that AMOTL1 is involved in activating Lats1 through promoting Merlin ubiquitination. This notion is consistent with the previous report (Kim et al. EMBO Rep 2015) that AMOTL2 activates Lats1/2. We agree with the reviewer that AMOTL1 ubiquitination induced by NEDD4L may also be involved in Lats1 activation. In this case, whether AMOTL1 ubiquitination activates Lats on its own or through activating Merlin warrants further study. A discussion of this possibility has been added on page 17.

Related to Figure 8 and S8:
Does NEDD4L modulate Hippo signaling in Meso-33 cells in the absence of ectopic expression of Merlin? If so, is this AMOTL1-dependent? Authors have only assessed BrdU and not provided any specific Hippo readouts.

We have examined the Hippo signaling in response to NEDD4L depletion in Meso-33 cells in the absence of ectopic expression of Merlin. The result showed that NEDD4L depletion does not affect Hippo signaling, indicated by phosphorylation of Lats1, YAP, and TAZ (Figure EV5D). Therefore, NEDD4L does not appear to modulate Hippo signaling in Meso-33 cells in the absence of ectopic expression of Merlin. A description of this result has been added on page 14.

In addition to BrdU, we have also examined the Hippo signaling in Meso-33 cells when ectopically expressing Merlin and its mutants. While wild-type Merlin induced the Hippo pathway activation, the K396R and S518D mutants showed a reduced ability to do so (Figure EV5C). A description of this result has been added on page 14.

Minor points:
In Figure S1B, why did authors only assess TAZ and not YAP?

We have also examined YAP and added the result in the revised Figure EV1C.

Figure S8 should be Figure S7.

We have fixed this labeling issue.

Referee #3:

Summary:
1-The manuscript reports a key finding: Merlin ubiquitination at K396 is central to the activation of Lats1 kinase and subsequently of the Hippo signaling pathway.
2-The finding is potentially of significance as it describes a new mechanism for NF2/Hippo activity regulation.
3- The work will be of interest to a large audience as the Hippo pathway is involved in a wide range of cellular functions and diseases.
4- The major finding is robustly documented.

Comments:

The manuscript by Wei and colleagues uncovers a new regulation mechanism of the activity of the NF2/Merlin tumor suppressor. The study clearly shows that Merlin is ubiquitinated on lysine 396 by the ubiquitine ligase NEDD4L. Access of NEDD4L to Merlin is provided by the scaffold AmotL1. Merlin ubiquitination facilitates the interaction with the kinase Lats1 for Hippo pathway activation. This prevents Yap/Taz nuclear localization and thus cell proliferation and tumor formation in nude mice xenograft model. In conclusion, the study claims that Merlin ubiquitination is a necessary step for Merlin activation of the Hippo pathway and, consequently, the inhibition of cellular growth.
The study is well organized and of good quality. The experimental design is sound. The results are convincing and will be of interest for the community of scientists working on NF2 and Hippo signaling.
This interesting work would, in my opinion, deserve publication in Embo report at the condition that the authors address three important questions in greater details as well a few minor points:

Major Points:
I-How relevant is the proposed mechanism for growth control?

a) Merlin ubiquitination is observed here under selected growth conditions (Calcium signaling and loss of adhesion) in a set of cell lines. Given that the most striking cellular phenotype associated to NF2 inactivation is the loss of contact inhibition of growth, could the author also investigate Merlin ubiquitination in this context? For example (but these are suggestions only), the authors could:

- Provide kinetics of Merlin ubiquitination upon increasing cell density over 48 hours.
- They could compare Merlin WT and K396R impact on cell growth and saturation density.

We have examined Merlin ubiquitination in the context of contact inhibition upon increasing cell density over 48 hours. However, we did not see a clear trend of changes in the ubiquitination (Figure R1[Figures for referees not shown.] , please see the response to Reviewer 2 on page 6 in this letter). These results suggested that the mode of Merlin regulation described in this

manuscript may not occur in the context of contact inhibition. Notably, contact inhibition appears to be a slow and progressively developed phenomenon, which usually occurs over several days. In contrast, cell detachment and Ca^{2+} signaling as tested here usually occurs within 30 minutes to several hours. Such a difference in time scale suggested that the mode of Merlin regulation may be more prominent or more readily detectable when the regulation process occurs relatively quickly.

b) From the figures, it appears that only a very small pool of Merlin is ubiquitinated. This is in strong contrast to the phosphorylation of Merlin on S518 that usually concerns most Merlin molecules. Only combined overexpression of NEDD4L and AmotL1 leads to a more robust signal of the ubiquitinated Merlin species (Figure 4F). However, the impact of Merlin ubiquitination on Lats1 binding and activation is very strong (Figure 6B, D and 7C). We would then expect Lats1 to be in complex preferentially with ubiquitinated Merlin.

- Quantification of the percentage of monoubiquitinated Merlin vs unmodified Merlin should be provided in several figures (1A, C, D, 2G etc... everywhere a conclusion is derived from an observed change in Merlin ubiquitination.

We have quantified the ratio of mono-ubiquitinated Merlin to the main Merlin band with each related western blot. The ratio is now shown under each blot panel.

- Proximity Ligation Assays are an acceptable way to show proximity but interaction should be validated by additional techniques like co-immunoprecipitation of Merlin and Lats1 .

We have tried to validate the interaction by performing co-immunoprecipitation using the LN229 cells expressing recombinant Merlin that we used in the Proximity Ligation Assays (PLA). However, we were unable to detect Lats1 from the precipitates co-immunopurified with Merlin. Of note, the recombinant Merlin in the above cells was stably expressed at a moderate level. When Merlin was transiently expressed at a much higher level, we can detect Lats1 from the co-immunoprecipitate with Merlin. In this latter case, we found that the amount of co-immunoprecipitated Lats1 was not regulated by thapsigargin, suggesting the Merlin-Lats1 interaction detected in these latter Merlin-overexpressed cells may not reflect physiological binding. Given this situation, we were unable to further evaluate Merlin's mutants. Therefore, when Merlin was moderately expressed, its interaction with Lats1 can be detected by PLA but not co-immunoprecipitation. This is presumably due to a higher sensitivity of PLA in detecting the protein-protein interaction or due to the co-immunoprecipitation condition being incompatible with the conditions that are required to preserve the interaction. Of note, for the Merlin-EBP50 interaction, we also found that it can be detected by PLA but not by the co-immunoprecipitation procedure in the same cells. Due to the above reasons, we used PLA in our experiments.

- More importantly, the author should evaluate the ratio of ubiquitinated vs unmodified

Merlin bound to Lats1. The relative abundance of the two Merlin species should be measurable by western-blot following coIP.

We have evaluated the ratio of ubiquitinated versus unmodified (native) Merlin bound to Lats1 following the reviewer's suggestion. The result has been added in Figure EV4F. After immunoprecipitating Lats1, we found that although both native (asterisk) and ubiquitinated (arrowhead) Merlin co-precipitated with Lats1, densitometric quantification indicated that the ratio of the upper band (arrowhead) to the lower band (asterisk) in the co-immunoprecipitated product was more than that in the lysate (Figure EV4F, lane 5, 0.38 vs 0.17). Such enrichment of the ubiquitinated Merlin in the co-precipitated product suggested that Lats1 binds more strongly to ubiquitinated Merlin. A description of this result has been added on page 13.

c) In addition to AmotL1, Amot and AmotL2 also bind to Merlin. Besides NEDD4L, the motin family was shown to interact with NEDD4 and Itch of the NEDD4 family (Wang et al Biochem. J. (2012) 444, 279-289). Are the observations presented in the manuscript relevant only for AmotL1 and NEDD4L?

- Could the authors test as proof-of-principle the effect of Amot, AmotL2, NEDD4 or Itch on Merlin ubiquitination? An experimental approach similar to Figure 3F would be sufficient.

We have used an experimental approach similar to Figure 3F to test the effect of AMOT, AMOTL2, NEDD4, and Itch on Merlin ubiquitination. We observed that upon co-expression with AMOTL1 and Merlin, both NEDD4 and Itch induce Merlin ubiquitination (Figure EV2G and EV2H). This observation suggested that NEDD4 and Itch may have similar functions to NEDD4L in this condition. In addition, we found that overexpression of AMOT or AMOTL2 with NEDD4L was able to induce Merlin ubiquitination similarly to AMOTL1 (Figure EV3D), suggesting that the AMOT family proteins may have similar properties in this setting. These results suggested that inducing Merlin ubiquitination may not be a NEDD4L- or AMOTL1-specific function. A description of this result has been added on pages 8 and 9.

- Furthermore, the endogenous levels of AmotL1 need to be presented when siRNA or shRNA are used (Figure 4A and 4B).

We have added the blotting result showing the endogenous levels of AMOTL1 when siRNA or shRNA are used in Figure 4A and 4B.

- Likewise, the endogenous levels of NEDD4L and AMOTL1 need to be documented in Figure 1A.

We have added the blotting result showing the endogenous levels of NEDD4L and AMOTL1 in Figure EV1A.

d) Merlin was previously shown to trigger Amot degradation via NEDD4. In the context

of the manuscript, this could represent a feedback mechanism to limit Merlin/Lats1 activation.
- Could the author evaluate the impact of Merlin WT, 518D and K396R expression on AmotL1 levels(e.g. with the cells used to generate Figure 8 H-K)?

We have examined the impact of Merlin WT, 518D, and K396R expression on AMOTL1 levels in FC1801 cells. The result showed that ectopic expression of Merlin did not affect AMOTL1 levels in these cells (Figure EV5F). A description of this result has been added on page 16.

II-How specific on Lats1 interaction is Merlin ubiquitination?

Lats1 binds to the FERM domain of Merlin like several other interactors. The authors could test how specific is the impact of ubiquitination on FERM domain interactors. For example:
- How do EBP50, alpha-tubulin or CD44 bind to Merlin K396R compared to Merlin WT or S518A upon cotransfection with NEDD4L and AmotL1.
- How does the C-terminal domain of Merlin co-IP with Merlin K396R compared to Merlin WT or S518A upon cotransfection with NEDD4L and AmotL1.
These experiments could help to discriminate between a specific regulation of Lats1 binding or a more global change in Merlin conformation.

We have examined the interaction between EBP50 and Merlin as an example to discriminate between a specific regulation of Lats1 binding or a more global change in Merlin conformation. First, we performed a standard co-immunoprecipitation of stably expressed Flag-tagged Merlin and endogenous EBP50. However, EBP50 was not detectable in the co-immunoprecipitated product (data not shown). Second, we conducted the Proximity Ligation Assay (PLA) using the same cells, and found that a PLA signal can be readily detected (Figures EV4C and EV4D). The distinct results from these two approaches appeared to mirror the situation of examining Merlin and Lats1 interaction as discussed above in addressing Comment I-b of this reviewer. The PLA result indicated that the Merlin and EBP50 binding is not enhanced by thapsigargin, nor is it inhibited by the K396R mutation (Figures EV4C and EV4D). This is in contrast to Merlin and Lats1 interaction (Figures 7A-7D). These results suggested that the impact of ubiquitination may not apply to all Merlin FERM domain binding proteins. A description of these results has been added on page 12.

III- How does this new mechanism fit with already known regulation of Merlin activity?

From the literature, it appears that a compact conformation of Merlin opens upon binding to PIP2 (Chinthalapudi et al 2018 Nat. Comm.). Phosphorylation of S518 doesn't appear to modify Merlin conformation but to inhibit the interaction with AmotL1 (Li et al 2015 cell research). Hence, as the authors discussed, Merlin ubiquitination appears as a step of a chain of events allowing Lats1 activation and cell growth inhibition.

However, to better convey the general message of the manuscript, it would be useful to

provide a scheme that put the main finding of the study (i.e. Merlin ubiquitination and impact on Lats/Hippo) into the context of what is already known about the mechanisms of Merlin regulation (e.g. in Figure 4C).

We have provided a scheme (Figure 8L) to illustrate our current finding in the context of what is known about the mechanisms of Merlin regulation. A discussion of this model has been added on pages 15 and 16.

Minor points:

1.) Subcellular localization of Merlin K396R is never shown. IF and /or cell fractionation should be presented in comparison to S518A (membrane enriched) and S518D (cytosol enriched). One cannot exclude that K396R mutation alters subcellular localization. Also, using fractionation, it would be possible to evaluate the distribution of ubiquitinated Merlin and refine the model.

To examine whether Merlin ubiquitination affects its membrane association, we conducted subcellular fractionation analysis. Interestingly, ubiquitinated Merlin was largely in the membrane fraction (Figure 7H). While NEDD4L localized in both cytosolic and membrane fractions, AMOTL1 was mostly in the membrane fraction. Merlin's cytosolic and membrane distribution was not affected by its K396R and S518A mutations, although the S518D mutant was slightly more cytosolic (Figure EV4G and EV4H). These results suggested that Merlin may be ubiquitinated at the plasma membrane, where it binds to Lats1. A description of these results has been added on page 13.

2.) There is no indication of the expression level of Merlin WT, 518D or K396R in the cells used in the sphere assay and the mouse tumor assay. This is necessary.

We have examined the expression of Merlin and its S518D or K396R mutants in FC1801 cells used in both assays and added this result in Figure EV5F and on page 14.

3.) The duration of cell suspension is not mentioned and should be clarified in the Material and Methods section. Does ubiquitination increase with time? Does it parallel with Lats1 phosphorylation?

We have clarified the cell suspension experiment in the Material and Methods section on page 18. We have examined Merlin ubiquitination and the Hippo signaling as cells gradually attached. The result showed that Merlin ubiquitination decreases over the course of attachment. It parallels Lats1 and YAP phosphorylation (Figure EV4A). A description of these results has been added on page 11.

4.) The tumor sphere assay is not really detailed. I guess the authors are using an agarose layer to prevent adhesion?

We have added more details for the tumor sphere assay in the Material and Methods section on page 23. The cells were cultured without agarose. In this condition, they can grow without attaching to the plate.

5.) The paper from K. Chinthalapudi et al. (2018. Lipid binding promotes the open conformation and tumor-suppressive activity of neurofibromin 2. Nat. Comm.) relative to merlin activation by PiP2 binding should be cited. As well as Li et al 2015. Angiomotin binding-induced activation of Merlin/NF2 in the Hippo pathway. Cell research) showing the opening and activation of Merlin via the binding to motins.

We have cited these two papers on pages 15 and 16.

Conclusion:
The study has the potential to be very interesting to a broad readership. However, in my opinion, it needs to address a couple important complementary questions in order to strengthen the relevance of the findings. Not all the suggested experiments need to be performed necessarily but the main points should be addressed.

1st Revision - Editorial Decision 15th Sep 2020

Dear Dr. Li,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, all three referees now support the publication of your study in EMBO reports.

Before we can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised manuscript.

- I would suggest this shortened title:
NEDD4L-mediated Merlin ubiquitination facilitates Hippo pathway activation

- Please name the 'summary' 'abstract'

- Please reduce the number of keywords. We can accommodate up to 5 key words.

- Please make sure that the funding information added in the online submission system is complete and similar to the one in the manuscript. It seems presently the funding information for the 'Four Diamonds Fund for Pediatric Cancer Research' is missing in the online form.

- Please add title to the legends of the EV figures (see also the attached word file - see below).

- Please add scale bars to Fig. 8H.

- Please order the panels in Fig. 1A such that the boxes in the first row have the same start and ending.

- There are still a couple of typos and grammatical errors present (see also the report of referee #2). Please have the final manuscript carefully proofread by a native speaker.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please not this file is not identical with the most recent version of the manuscript (it e.g. lacks the DAS and the mass spec section). Please just use it as indication what to correct in your most recent manuscript file. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Kind regards,

Achim Breiling
Editor
EMBO Reports

Referee #1:

The authors have addressed adequately all the reviewers concerns. I would consider that it could be accepted at this point.

Referee #2:

The authors addressed the vast majority of all the reviewers' comments either experimentally or by modifying the manuscript. I believe the manuscript is significantly enhanced from the original version and its discussion of the new findings within the global context or Merlin and Hippo regulation is more thorough.

Only minor text changes (typos and grammar) are required before publication.

Referee #3:

The authors have responded to virtually all my requests. They have added a very significant amont of new data. The answers are satisfactory and strongly improve the quality of the manuscript and the strength of the conclusions. They also raise new questions but these go beyond the scope of the manuscript. I think it is an interesting study that uncovers new mechanisms of regulation of Hippo pathway by the tumor suppressor Merlin. It will then be of interest to a large audience and should foster new investigations in this field.

At this point, I recommend the manuscript for publication in EMBO Reports as it is.

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 18th Sep 2020

Dr. Wei Li
Penn State Hershey College of Medicine
Department of Pediatrics
500 University Drive
PO Box 850, MC H085
Hershey, PA 17033
United States

Dear Dr. Li,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

****

THINGS TO DO NOW:

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2020-50642V3 and be addressed to emboreports@wiley.com.

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

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Wei Li
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2020-50642V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Samples sizes were chosen based on if the differences between groups are biologically meaningful and are statistically significant. (page 23)
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Estimation of sample size for the animal studies was based on previous studies using the same tumor model, but not based on certain statistical method. (page 23)
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No smaples were excluded. (page 24)
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Yes. Animals were from the same cohort. Animals were randomized when tumor cells from different groups were implanted. All animals were maintained in the same environment and handled by the same procedure. (page 24)
For animal studies, include a statement about randomization even if no randomization was used.	Animals were randomized when tumor cells from different groups were implanted. (page 24)
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The investigators were not blinded to group allocation during data collection and/or analysis. (page 24)
4.b. For animal studies, include a statement about blinding even if no blinding was done	The investigators were not blinded to group allocation during data collection and/or analysis. (page 24)
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	No.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies were from commercial sources and have been used in the literature. Vendor and catalog number for each antibody has been provided. (page 18)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The source of cell lines, if they were recently authenticated and tested for mycoplasma contamination have been reported in Material and Methods. (page 18)

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Species, strain, gender, age, source, housing and husbandry conditions of animals have been reported in Material and Methods. (page 17)
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experimental protocols were approved by the Penn State University Institutional Animal Care and Use Committee. This have been reported in Material and Methods. (page 17)
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	All methods were performed in accordance with the relevant guidelines and regulations. This have been reported in Material and Methods. (page 17)

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The mass spectrometry data from this publication have been deposited to the ProteomeXchange Consortium via the PRIDE (https://www.ebi.ac.uk/pride/) partner repository with the dataset identifier PXD021057 and PXD021058. (page 25)
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA