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Phosphorylation by NLK inhibits YAP-14-3-3-interactions and induces its nuclear localization

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(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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 08 June 2016

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 three referees acknowledge the potential interest of the findings. However, all three referees have raised a number of concerns and suggestions to improve the manuscript or to strengthen the data and the conclusions drawn. In particular all points by referee #1 are important. Also most concerns of referees #2 and #3 need to be addressed. However, we feel that points 5 (additional NLK inhibitor), 13 (cell density), 16 (other cell lines) and 19 (Nemo levels affect Yorkie phosphorylation) of referee #2 and point 9 of referee #3 do not need to be addressed experimentally (however, if you can address these, it would certainly strengthen the manuscript further). Most importantly, proper quantifications and statistics should be provided where applicable throughout the manuscript!

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns (as detailed in their reports) must be fully addressed in a complete 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 or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

Referee #1:

Moon et al. describe in this interesting manuscript the identification of a novel regulatory mechanisms of the Hippo signalling effector YAP. More specifically, by describing and studying the NLK-mediated phosphorylation of YAP on Ser128, Moon et al. uncover how regulatory Ser127 phosphorylation appears to be influenced. While the work is well-presented and the overall implications of the research findings have great potential, I must unfortunately state that the manuscript in its current format has some significant shortcomings that are listed below.

Main points:

1) Evidence for S128 and S127 phosphorylation being mutually exclusive
The authors state in the abstract and in other sections that Ser128 phosphorylation by NLK and Ser127 phosphorylation by Lats kinases are mutually exclusive. However, this aspect has not been really tested and currently is more speculative than anything else. Therefore the authors should:

(i) Perform the following in vitro kinase assays:

-phosphorylate recombinant YAP with NLK, then remove NLK and subsequently test the S128 phosphorylated YAP as substrate for Lats kinase - I would recommend to focus on S127 and S128 using their anti-phospho antibodies, and it will be important to include proper controls such as S127A, S128A, S127A/S128A and S128D vs. wild-type - the key question here is: can Lats phosphorylate on Ser127 irrespective of the S128 phosphorylation status in vitro?

-phosphorylate recombinant YAP with Lats, then wash out Lats and subsequently test Ser127 phosphorylated YAP as substrate for NLK - the reciprocal experiment for the one outlined above - the key question here is: does NLK phosphorylate YAP on Ser128 irrespective of the S127 phosphorylation status in vitro?

(ii) Analyse Ser381/Ser397 of YAP by Lats kinases: Considering that the phosphorylation of YAP on Ser381 (sometimes also termed Ser397, depending on the YAP isoform analysed) by Lats actually regulates the proteasomal degradation of YAP (i.e. Zhao et al., 2010), I think that it would also be important to complete at least a few key experiments, such as the in vitro kinase assays and other panels presented in Figure 2, with also including anti-S381/397 immunoblots. The commercially available antibody from Cell Signaling would represent an excellent tool to tackle this important point. Furthermore, this extension to a second phosphorylation site on YAP by Lats kinases has the great potential to further strengthen the observation that the NLK mediated phosphorylation of YAP on S128 only selectively affects the S127 and not another site on YAP.

2) Improve/extend introduction section: I think in order to give the non-expert reader a more comprehensive overview of the Hippo pathway and its regulation, the authors should:

(i) Cite all five Hippo/dMst references from 2003, since also *Nature Cell Biology* and *Genes & Development* are very respected journals (just focusing on *Cell* would be rather strange). I know that you will face reference limitations, hence maybe some reviews can be removed and original research articles added instead.

(ii) Clearly state the regulatory difference between S127 and S381/S397 phosphorylation of YAP, with the corresponding references.

(iii) Mention also TAZ as Hippo effector that is regulated by Lats kinases.

3) Extension of Figure 1.
To fully support the conclusions drawn from Figure 1, the authors need to expand Figure 1B as follows: perform kinase assays with NLK kinase alone and recombinant YAP alone in addition to

the two lane experiment shown in Figure 2B. The current evidence does not exclude the possibility that the shown band is a result of the autophosphorylation of NLK (or potentially a kinase/substrate that was co-immunoprecipitated with NLK).

4) Extension of Fig. 2: To fully support their conclusions regarding this figure, the authors need to:

(i) Expand panel 2B as outlined above in point 1 by also probing for S381/397, and also show the quantification of three independent experiments as a histogram (n=3 is needed here).

(ii) Expand panel 2C as outlined above in point 2 by also probing for S381/397, and show the quantification of three independent experiments, and show the results of two independent siRNAs targeting NLK.

(iii) Expand panel 2D and include S381/397 phosphorylation, and very importantly study S127, S128 and S381/397 on endogenous YAP.

(iv) Regarding panel 2E: how can the authors be certain that observed differences are not a result of epitope masking of the anti-phospho antibodies tested here. Potentially the anti-S127-P antibody cannot properly detect YAP when it is phosphorylated on S128 and vice versa. Therefore, the in vitro kinase assays as outlined above in point 1 are essential to ensure that this is not case.

5) Extension of Fig 3: As for figures 1 and 2 (and actually all the main figures) also this figure needs to be extended to fully support the conclusions drawn by the authors:

(i) Figure 3A needs to also show the anti-HA blot for the IP to be completed on the exogenous level. Even more importantly, does the knockdown of endogenous NLK also affect this complex formation?

(ii) Please explain why the experimental co-IP set up was changed from 3A to 3B.

(iii) Please extend Figure 3C/D with a biochemical nuclear/cytoplasm fractionation to fully establish this very interesting point.

6) Extension of Figure 4.

(i) In Figure 4B the authors should also seriously consider an extension that allows conclusions regarding the activity status of NLK. They could either check for a phosphorylation on NLK that correlates with NLK activity or measure the phosphorylation of an established NLK substrate, besides YAP.

(ii) Figure 4C should also include the analysis of S127 phosphorylation (if possible S381/397 phosphorylation would also be interesting in this context).

7) Extension of Figure 5.

(i) Regarding NLK mediated S128 phosphorylation of YAP and then the YAP/TEAD interaction, I think that the authors should extend Figure 5A and also determine how S128A and S128D bind to TEAD.
(ii) Expand Figure 5G to fully link S128 phosphorylation to NLK in the context of cell migration. Does YAP S127A and S128D rescue the decreased migration of NLK knockdown cells?

8) Confirm that Yorkie target gene expression reduction upon NLK knockdown is Yorkie dependent. Is there a quick way to confirm that observed differences in Nemo deficient tissue are Yorkie dependent. For example does Hippo insensitive Yorkie rescue the expression in Nemo deficient tissue?

Minor points:

A) Mention other YAP S127 kinases: Considering that it was recently reported that YAP can also be regulated by S127 phosphorylation mediated by the Ndr kinases (see Zhang et al., 2015), the authors maybe also want to mention this briefly in the discussion section. Potentially, NLK mediated

phosphorylation of YAP affects more than the Lats mediated phosphorylation of YAP. - I do not expect the authors to test this experimentally, but maybe it is worth mentioning (if space allows).

B) Extend a little bit more on S127 related literature: In order to give a broader picture regarding the role of S127 phosphorylation of YAP, I think that the authors should also mention and reference the following key publication: Chen Q, Zhang N, Xie R, Wang W, Cai J, Choi KS, David KK, Huang B, Yabuta N, Nojima H, Anders RA, Pan D. Homeostatic control of Hippo signaling activity revealed by an endogenous activating mutation in YAP. Genes Dev. 2015 Jun 15;29(12):1285-97. Doi 10.1101/gad.264234.115. PubMed PMID: 26109051; PubMed Central PMCID: PMC4495399.

C) Re-phrase sentence on page 4: Please re-phrase the following: "... indicating NLK-mediated YAP phosphorylation was responsible for the mobility shift ..." The authors should note that the data shown at this stage of the manuscript in the first figures do not allow them to draw this conclusion here. These experiments rather show that YAP phosphorylation can be influenced the kinase activity status of NLK.

D) Re-phrase sentence on page 5: Please rephrase: "To examine whether NLK directly phosphorylates Ser128 of YAP, ..." Again this is here a premature conclusion, since the co-expression in cells does not allow the conclusion of a direct phosphorylation. Direct phosphorylation is tested by in vitro kinase assays using recombinant proteins.

E) Check for NLS close to S128 phosphorylation site: Could the observed localisation of S128D be a consequence of influencing a NLS that is close by of the S127/S128 site?

F) Be careful with the wording on page 6: The authors do not really measure "the stability" of NLK, so I would re-phrase the text accordingly.

G) Consider testing nuclear targeted NLK: Based on the model that is proposed on page 9, the authors maybe want to test the consequences of permanently targeting NLK to the nucleus. - not an essential point, but this would further strengthen their proposed model.

Referee #2:

The manuscript by Moon and colleagues presents an interesting and novel angle on the regulation of the transcriptional co-activator YAP. The authors suggest that NLK interacts with and directly phosphorylates YAP at Serine 128, and provide evidence that this modification promotes nuclear YAP localization and activity. Serine 128 within YAP lies adjacent to the well characterized Serine 127 that is phosphorylated in response to activation of the Hippo pathway. The authors suggest that modification of these residues act in an antagonistic manner to the other. The authors show data supporting their model, including data that shows NLK-induced p-S128 YAP reduces phosphorylation of YAP on S127. The authors also show that a S128A mutant of YAP more strongly interacts with 14-3-3 and is localized more prominently in the cytoplasm of HeLa cells.

The authors propose an interesting new mechanism for the regulation of YAP, and identify a new post-translational modification that impacts YAP activity. However, while there is some novelty to proposed mechanism, major experimental and conceptual issues exist, particularly with respect to the relevance of NLK in the proposed mechanism. Overall, the data in the manuscript need significant improvements, and more experimental support for their proposed model in vitro and in vivo are required before publication. Some comments that need to be addressed are list below:

1) - A crucial control for the kinase assay conducted in Fig 1B is testing whether purified GST is a target of NLK. This kinase assay should be conducted in parallel with GST-YAP and the kinase-dead versions of NLK. Moreover, an important experiment to validate whether NLK directly phosphorylates S128 in YAP is the test whether NLK phosphorylates the S128A mutant of YAP in vitro.

2) - The data in Fig 2B is not convincing to show that NLK reduces YAP phosphorylation on S127. In particular, the quantitation included should show data from at least three experiments and include error bars and statistics. Also, it is curious that NLK expression in this figure does not induce a mobility shift in endogenous YAP. Related to the prior point, why is there no mobility shift in YAP when co-expressed with NLK in Figures 2D and EV2B? This is inconsistent with the data shown in Fig 1A.

3) - The figure legend for Fig EV2B seems to have an error, as it makes reference to phospho-S127 YAP quantitation.

4) - The authors make the statement that their data "suggest that LiCl-mediated induction of YAP phosphorylation of Ser127 was due to inhibition of NLK". LiCl may inhibit many kinases beyond GSK3b and therefore a conclusion about NLK from this experiment cannot be made. This statement needs to be revised, and if this data is included, a way of better linking this experiment to NLK should be considered. For example, can LiCl inhibit the effects of NLK overexpression? Can LiCl affect p-S128 YAP levels?

5) - The knockdown of GSK3b in Fig EV3B is very minimal. Based on this data, the authors cannot make any statements about whether GSK3b affects phosphorylation of YAP on S127. As alluded to in the prior point, using LiCl as a means to assess NLK activity is not ideal and I would suggest using alternative methods to inhibit NLK activity.

6) - The quantitation in Fig 2C should show data from at least three experiments and include statistics.

7) - The data in Fig 2E is not convincing for the conclusions made by the authors. The total levels of WT and S128A YAP are not equivalent and the increased levels of S128A YAP may explain why there is an observed higher level of p-YAP(S127). Also, it is difficult to make a strong conclusion about p-YAP(S128) given the non-specific band. The authors do not seem to observe a similar non-specific band in Fig EV2B, so it seems possible to improve this data.

8) - While the authors show that the pS128 antibody recognizes overexpressed wild type YAP, but not S128A-YAP, the authors do not provide any controls showing that the antibody recognizes endogenous YAP. The authors should include a knockdown experiment of YAP showing that this antibody is specific for endogenous YAP.

9) - It is unclear why reference is made to Flag-NLK in the Fig 3B image.

10) - It is unclear why the authors examined the localization of GFP-tagged YAP in Fig EV4. The authors should test whether NLK expression induces endogenous nuclear Yap localization. Also, all experiments showing YAP localization changes should include quantitation, as a small field of view may not represent the overall changes.

11) - The quantitation in Fig 4B should show data from at least three experiments and include error bars. Also, the levels of pS127-YAP and p-S128-YAP should be shown in this experiment.

12) - An IgG control needs to be included in the IP experiment shown in Fig 4C to rule out non-specific bands, particularly given that the p-S128 antibody is shown to have non-specific bands associated with it.

13) - It is interesting that NLK exhibits similar changes in localization as seen with YAP upon cell density changes. However, it is unclear what this data means in the context of NLK function. Why is this important? Does NLK interact with YAP only in the nucleus? If so, what is the significance of this occurring under low density conditions? For example, is p-S128 modification of YAP important for its cell proliferation roles, or it's transcriptional activity? How is NLK cytoplasmic localization induced upon cell density, and why are NLK levels reduced? Is NLK regulated by the Hippo pathway? Without some kind of mechanistic insight into how density affects NLK activity on YAP, Fig 4 does not add any information that is related to YAP.

14) - An IgG control needs to be included in the IP experiment shown in Fig 5A to rule out non-

specific bands. This is very important for detecting proteins such as TEAD1, as the protein runs almost exactly with the heavy chain of the antibody used for the IP. Also, the levels of pS128-YAP should be included in the analysis of the lysates.

15) - The authors should correct the y-axis in Fig 5C as the data is from a qPCR experiment.

16) - The effects of NLK knockdown on expression changes of YAP target genes was assessed only in HEK-293T cells. It would strengthen the paper to show data from a similar experiment in another cell line.

17) - Many of the experiments throughout the paper use different cell lines, switching between mouse and human and epithelial and fibroblast. No justification for switching cell lines for the different experiments is given. Also, no information on whether NLK is expressed and to what level it is within these different cell lines is given. The use of each cell line and the rationale should be included in the manuscript.

18) - The data in Fig 5D is not convincing. Based on the image shown, it does not appear that nmo-RNAi leads to reduced expanded levels. Similarly, the images in Fig 5E are also not convincing. Moreover, to draw conclusions from these in vivo experiments Nemo levels need to be verified that they are being knocked down or induced.

19) - Antibodies exist for examining Yorkie phosphorylation in Drosophila, and therefore to validate the mechanism proposed by the authors in this model system the authors should show that alterations in Nemo levels affects Yorkie phosphorylation. The authors should also test whether the conserved S128 residue regulated Yorkie in the same way as it does in YAP. If the p-S128 YAP antibody recognizes Yorkie, it could be used to experimentally to examine whether Nemo regulates Yorkie.

20) - It is unclear from the cell migration assays in Fig 5F how this relates to YAP. While it is interesting that NLK knockdown reduces cell migration, there are a multitude of ways this could be achieved and therefore no relationship to YAP, or phosphorylation of YAP, can be drawn from this data.

21) - More details for how relative width was calculated from area measurements in Fig 5G should be included. Importantly, for these experiments, parallel analyses of the levels of the various YAP mutants needs to be determined so that any differences can be attributed to the mutation rather than the differences in expression.

Referee #3:

Moon et al., report NLK as a kinase that phosphorylates Yap and activates it. Their data lend some support to a mechanism whereby NLK-mediated S128 phosphorylation activates YAP by preventing LATS1/2-mediated phosphorylation of S127. The studies uses biochemistry, human cultured cell assays and some fly genetics to make a reasonably convincing case. The physiological context in which this phosphorylation occurs is not explored. The manuscript has promise but many things need substantial attention, including some key experiments that address how important NLK regulation of YAP is. Further, the manuscript relies mostly on overexpression tissue culture studies. Loss of function studies are crucial. The conclusions don't match the data at present and more detailed analysis of endogenous NLK is essential.

1) In Figure 1C the NEK/YAP is not overly convincing. Have these been proteins ben found to interact in the many unbiased proteomic studies that have been performed with YAP?

2) Figure 2- Does the P-S128-YAP antibody detect endogenous P-S128-YAP? Two important experiments are missing that relate to the importance of NLK-mediated phosphorylation of YAP:

1. does depletion or CRISPR-mutation of NEK reduce/ablate P-S128-YAP phosphorylation?

2. does depletion or CRISPR-mutation of NEK increase P-S127-YAP phosphorylation by LATS1/2?

These sorts of experiments are important to determine how important NEK is for YAP regulation. SO far this is not addressed at all in the manuscript.

3) Figure 3 - Does NLK LOF increase YAP/14-3-3 binding and YAP localization in cells?

4) Figure 4 - 4C is a key result. It should be quantified from multiple experiments and more of the P-YAP-S128 blot should be shown. Why do you think there are multiple bands here?

5) Can NLK siRNA increase YAP-14-3-3 interaction in low density cells?

6) Figure 5 - the IPs in 5A are not convincing. Show more of the blots.

7) The wound assays in 5G need to be repeated and quantified. Does S128A close the wound faster than WT? If not, then what is the importance of NLK? AT preswnt hteis claim is unfounded without quantification: "Wound healing ability was affected by the phosphorylation status of Ser127 or Ser128."

8) The data in Drosophila tissues are important because without in vivo experiments the impact of this mostly cell culture based study is far less. However these studies are presently weak and require substantial improvement to be convincing. The current expanded-lacZ stainings seem to skim the top of the disc and need to come from the disc proper. It is quite possible that Nemo modulation causes a morphological change to the disc and that is what we see in Fig 6, rather than a change in expanded-lacZ levels.

The authors should do the following:

i: capture images that are clearly from the middle plane of the disc, rather than the apical or basal surface and ii: Capture a X-Z section. And also image one or more independent readouts of Hippo pathway activity, such as bantam or DIAP1, to validate the expanded-lacZ experiments.

9) What about epistasis experiments? These could really help to strengthen the manuscript.

10) The authors should far more of some Western blots - e.g. Fig 4C, 5A and 1C.

1st Revision - authors' response 22 September 2016

Thank you very much for your interest in our work and for providing us with the opportunity to submit a revised version of our manuscript entitled "NLK phosphorylates and potentiates YAP activity by regulating interaction with 14-3-3 and nuclear localization". We also would like to thank the reviewers for their thoughtful and constructive comments, which have greatly helped us immensely to improve our study. We have performed additional experiments and revised our manuscript in accordance with the reviewer's suggestions. We strongly believe that we have adequately addressed all the points raised by and to the satisfaction of the reviewers. To facilitate the assessment of our revision, we have provided point-by-point responses herein. Also, we have incorporated our responses into the revised manuscript and updated the figure numbering to reflect the changes we have made in response to the reviewer's comments.

Referee #1:

Moon et al. describe in this interesting manuscript the identification of a novel regulatory mechanisms of the Hippo signaling effector YAP. More specifically, by describing and studying the NLK-mediated phosphorylation of YAP on Ser128, Moon et al. uncover how regulatory Ser127 phosphorylation appears to be influenced. While the work is well-presented and the overall implications of the research findings have great potential, I must unfortunately state that the manuscript in its current format has some significant shortcomings that are listed below.

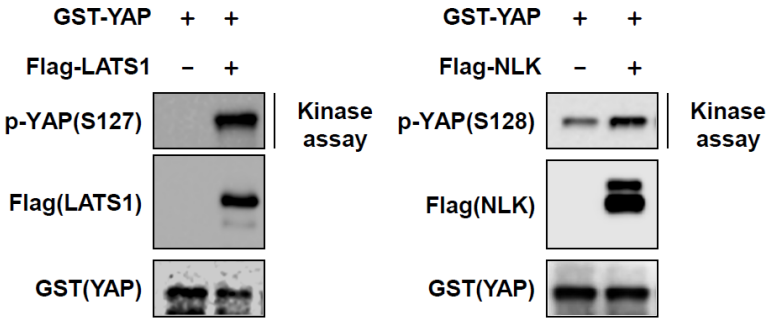
RESPONSE: We thank the Referee 1 for the constructive and thoughtful suggestions. We have fully addressed the reviewer's concerns as shown below.

Main points:

1) Evidence for S128 and S127 phosphorylation being mutually exclusive.
The authors state in the abstract and in other sections that Ser128 phosphorylation by NLK and Ser127 phosphorylation by Lats kinases are mutually exclusive. However, this aspect has not been really tested and currently is more speculative than anything else. Therefore the authors should:

(i) Perform the following in vitro kinase assays: -phosphorylate recombinant YAP with NLK, then remove NLK and subsequently test the S128 phosphorylated YAP as substrate for Lats kinase - I would recommend to focus on S127 and S128 using their anti-phospho antibodies, and it will be important to include proper controls such as S127A, S128A, S127A/S128A and S128D vs. wild-type - the key question here is: can Lats phosphorylate on Ser127 irrespective of the S128 phosphorylation status in vitro?

RESPONSE: Thank you for suggesting these really thoughtful experiments. However, the experiments suggested by the reviewer - phosphorylate recombinant YAP with NLK, then remove NLK and subsequently test the S128 phosphorylated YAP as substrate for Lats kinase - was technically difficult to perform obtaining clean data for the following reason. In order to get good experimental data to address the reviewer's concerns, the activities of both kinases should be strong and pYAP-S127 and -S128 antibodies need to be very specific to the respective phosphorylated forms of YAP. However, as you see on the following figure, which is similar to the data obtained by Dr. Guan and colleagues in the accompanying manuscript, the increase of pYAP-S127 by Lats1 was very strong and it was specifically detected by pYAP-S127 antibody, whereas the increase of pYAP-S128 by NLK was weak and pYAP-S128 antibody also detected (although to a reduced degree) the unphosphorylated form of YAP-S128 in vitro. Actually, the pYAP-S397 antibody exhibits similar properties (see the answer for the response to (1)-(ii)). These data are presented only for the reviewer's evaluation.



Because the pYAP-S127 antibody is very specific, using this antibody we have tried to answer the question: Can Lats phosphorylate Ser127 irrespective of the S128 phosphorylation status *in vitro*? Immunoprecipitated Flag-Lats1 from lysates of HEK293T cells transfected with Flag-Lats1 was incubated with various mutant forms of GST-YAP. As shown in Fig EV3G, the phosphorylation of GST-YAP(S128D), NLK phosphor-mimetic form, by Lats1 was strongly reduced, which suggests that the phosphorylation of S128 by NLK inhibits phosphorylation of YAP-S127 by Lats *in vitro*.

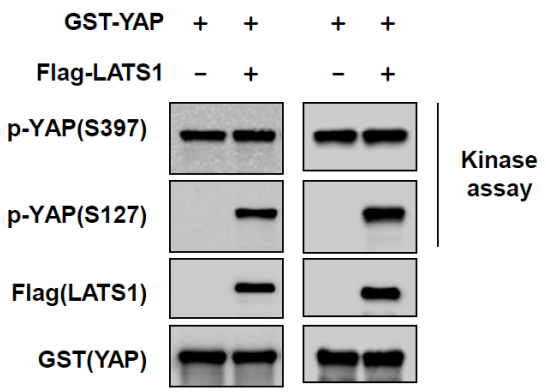
-phosphorylate recombinant YAP with Lats, then wash out Lats and subsequently test Ser127 phosphorylated YAP as substrate for NLK - the reciprocal experiment for the one outlined above - the key question here is: does NLK phosphorylate YAP on Ser128 irrespective of the S127 phosphorylation status in vitro?

RESPONSE: Please see our answer to the above question.

(ii) Analyse Ser381/Ser397 of YAP by Lats kinases. Considering that the phosphorylation of YAP on Ser381 (sometimes also termed Ser397, depending on the YAP isoform analysed) by Lats

actually regulates the proteasomal degradation of YAP (i.e. Zhao et al., 2010), I think that it would also be important to complete at least a few key experiments, such as the in vitro kinase assays and other panels presented in Figure 2, with also including anti-S381/397 immunoblots. The commercially available antibody from Cell Signaling would represent an excellent tool to tackle this important point. Furthermore, this extension to a second phosphorylation site on YAP by Lats kinases has the great potential to further strengthen the observation that the NLK mediated phosphorylation of YAP on S128 only selectively affects the S127 and not another site on YAP.

RESPONSE: Thank you for the constructive suggestion. Based on reviewer's suggestion we have performed in vitro kinase assay by incubating GST-YAP with immunoprecipitated Flag-Lats1 from lysates of HEK293T cells transfected without or with Flag-Lats1. The level of pYAP-S127 was increased by Lats1 as expected, but pYAP-S397 was not increased. More strangely, the pYAP-S397 specific antibody detected the pYAP-S397 band even without Flag-Lats1. Although the pYAP-S397 antibody is well characterized and specifically detects endogenous pYAP-S397, we think that this antibody may also detect the unphosphorylated form of YAP. With this technical limitation, instead of doing the in vitro kinase assays, we have focused on examining the level of pYAP-S397 in most cases of cell culture experiments (Fig 2B-D, Fig 4B and Fig 5A). Interestingly, we found that the level of pYAP-S397 was also reduced when wild-type NLK was overexpressed. Consistently, ablation of NLK increased the level of pYAP-S397. As we have stated in the revised manuscript, we think that the decrease in S397 phosphorylation might be due to the enhanced nuclear localization of YAP by NLK (Fig 3D and E, Fig EV4A), which sequesters YAP from phosphorylation by Lats1/2. Because LC-MS analysis could not reveal all phosphorylation sites due to the technical limitation, we may not have detected phosphorylation on S397 in Fig EV2A.



2) Improve/extend introduction section. I think in order to give the non-expert reader a more comprehensive overview of the Hippo pathway and its regulation, the authors should:

(i) Cite all five Hippo/dMst references from 2003, since also Nature Cell Biology and Genes&Development are very respected journals (just focusing on Cell would be rather strange). - I know that you will face reference limitations, hence maybe some reviews can be removed and original research articles added instead.

RESPONSE: Thank you for your suggestion. We have revised the manuscript as suggested by the reviewer.

(ii) Clearly state the regulatory difference between S127 and S381/S397 phosphorylation of YAP, with the corresponding references.

RESPONSE: We have now described the regulatory difference in the Introduction section.

(iii) Mention also TAZ as Hippo effector that is regulated by Lats kinases.

RESPONSE: We have now mentioned TAZ as a Hippo effector in the Introduction section

3) Extension of Figure 1: To fully support the conclusions drawn from Figure 1, the authors need to expand Figure 1B as follows: perform kinase assays with NLK kinase alone and recombinant YAP alone in addition to the two lane experiment shown in Figure 2B. The current evidence does not exclude the possibility that the shown band is a result of the autophosphorylation of NLK (or potentially a kinase/substrate that was co-immunoprecipitated with NLK).

RESPONSE: Thank you for the thoughtful suggestion. However, the band detected in the NLK-WT condition has the size of GST-YAP, which excludes the possibility that this band is a result of the

autophosphorylation of NLK. Because the possibility that Flag-NLK-WT and Flag-NLK-KM would interact with another kinase which can phosphorylate GST-YAP, or with a substrate of the same size as GST-YAP, is low, we think that the band is not a kinase/substrate that was co-immunoprecipitated with NLK.

4) Extension of Fig 2: To fully support their conclusions regarding this figure, the authors need to

(i) Expand panel 2B as outlined above in point 1 by also probing for S381/397, and also show the quantification of three independent experiments as a histogram (n=3 is needed here).

RESPONSE: We have performed three independent experiments including the pYAP-S397 readout and the quantification is shown in Fig 2B of the revised manuscript as reviewers have suggested. This issue was discussed above in response to the reviewer's comments (1)-(ii).

(ii) Expand panel 2C as outlined above in point 2 by also probing for S381/397, and show the quantification of three independent experiments, and show the results of two independent siRNAs targeting NLK.

RESPONSE: Regarding this concern, the reviewer 3 suggested adopting the CRSPR/Cas9 system to secure better and more reliable results. We have generated two different NLK KO pool cell lines. We have performed three independent experiments including the pYAP-S397 readout and the quantification is shown in Fig 2C of the revised manuscript as the reviewers have suggested. The data using NLK KO cell lines is comparable to the result shown in previous Fig 2C with NLK siRNA.

(iii) Expand panel 2D and include S381/397 phosphorylation, and very importantly study S127, S128 and S381/397 on endogenous YAP.

RESPONSE: Please see panel 2D. We have now performed Western blot analysis of the phosphorylation status, including S397 on endogenous YAP. Because of the logical flow of the manuscript, the data for endogenous level of pYAP-S128 after the overexpression of NLK (WT or KM) and NLK KO are shown in Fig EV3D and Fig 5A, respectively. The results for the endogenous pYAP-S127 and S-397 in cells NLK overexpressing cells are included in Fig 2B, and those in NLK knockout cells in Fig 5A.

(iv) Regarding panel 2E: how can the authors be certain that observed differences are not a result of epitope masking of the anti-phospho antibodies tested here. Potentially the anti-S127-P antibody cannot properly detect YAP when it is phosphorylated on S128 and vice versa. Therefore, the in vitro kinase assays as outlined above in point 1 are essential to ensure that this is not case.

RESPONSE: Thank you for this thoughtful criticism. We definitely agree that these data can be interpreted as a result of epitope masking. First of all we would like to summarize results related to phosphorylation of S127 or S128.

Result (1): Fig 2B and C, overexpression or KO of NLK reduces (or increases) S127 phosphorylation, which are detected by the pYAP-S127 specific antibody.
Result (2): Fig 2D, overexpression of NLK enhances S128 phosphorylation, which is detected by the pYAP-S128 specific antibody.
Result (3): Fig 2E, the S127A mutation enhances S128 phosphorylation, and the S128A mutation enhances S127 phosphorylation, which is detected by pYAP-S128 or pYAP-S127, respectively, specific antibody.
Result (4): Fig 3C, 14-3-3 shows weaker interaction with YAP-S127A than with the wild type YAP, while stronger interaction with YAP-S128A than with the wild type YAP.

When we consider all the above data, we think that the possibility of epitope masking may not explain our data, for the following reasons.
Results (1) and (2): The possibility of epitope masking can be applicable, however, the possibility that two independent antibodies- specific for S127 or S128 phosphorylation- are experiencing epitope masking may not be that high.

Result (3): In contrast to Results (1) and (2), which might have been a result of epitope masking due to phosphorylation of neighboring sites, Result (3) shows that mutation of serine to alanine enhances phosphorylation on the neighboring site, as detected with two independent antibodies. The Result (3) is difficult to reconcile with the idea of epitope masking.
Result (4): If the difference detected by S127 or S128 phosphorylation-specific antibodies were due to epitope masking without actual change of phosphorylation, the interaction between 14-3-3 and YAP mutants would not be changed.

Overall, although we could not provide strong direct evidence using in vitro kinase assays, our cumulative data strongly suggest that phosphorylation on S128 by NLK actually reduces phosphorylation level on S127.

5) Extension of Figure 3: As for figures 1 and 2 (and actually all the main figures) also this figure needs to be extended to fully support the conclusions drawn by the authors:

(i) Figure 3A needs to also show the anti-HA blot for the IP to be completed on the exogenous level. Even more importantly, does the knockdown of endogenous NLK also affect this complex formation?

RESPONSE: We apologize for the omission and thank the reviewer for this thoughtful suggestion. We did not include the anti-HA blot by mistake and have now added the anti-HA blot to the IP panel in Figure 3A of the revised version. Also, using two different NLK KO cell lines, we carried out IP experiments and observed consistent results: the interaction between the endogenous YAP and 14-3-3 is enhanced when the level of NLK is reduced (Fig 3B).

(ii) Please explain why the experimental co-IP set up was changed from 3A to 3B.

RESPONSE: Flag-NLK should have been labeled as Flag-YAP in Fig 3B of the original version. We apologize for this mistake, which is now corrected (Fig 3C). Because we think that the reviewer's concern is due to our labeling mistake, we provide below (but not in the main text) an additional explanation of this experimental setup. Our aim was to test whether the NLK-mediated phosphorylation status of YAP on Ser128 affected the interaction between YAP and 14-3-3. To test whether different phosphorylations on YAP provide differential ability to interact with 14-3-3, we performed the IP experiment with various YAP phosphor mutant forms, and found that YAP-S128A showed a stronger interaction with 14-3-3 than YAP-WT did, whereas neither YAP-S127A nor YAP-S128D showed binding to 14-3-3.

(iii) Please extend Figure 3C/D with a biochemical nuclear/cytoplasm fractionation to fully establish this very interesting point.

RESPONSE: We thank the reviewer for bringing up this suggestion to strengthen our conclusion. We have performed subcellular fractionation and added new data in Fig EV4C of the revised version. Consistently, YAP-S127A or -S128D localized more in the nucleus than wild-type YAP did, while the NLK phosphor-deficient mimetic form (S128A) showed less nuclear localization than wild-type YAP did.

6) Extension of Figure 4:

(i) In Figure 4B the authors should also seriously consider an extension that allows conclusions regarding the activity status of NLK. They could either check for a phosphorylation on NLK that correlates with NLK activity or measure the phosphorylation of an established NLK substrate, besides YAP.

RESPONSE: We understand why the reviewer has brought up this point. Unfortunately, there are no commercially available antibodies either to detect the activity of NLK or the phosphorylation status of NLK substrates including LEF1, DP1 and NICD. More importantly, the critical point shown in Fig 4B is that the NLK protein level is regulated in a cell density dependent manner. The level of NLK protein is down-regulated in high cell density of multiple cell lines, along with reduced NLK mediated phosphorylation of YAP-S128. We think that resolving this issue raised by the reviewer is not absolutely necessary to strengthen our conclusions.

(ii) Figure 4C should also include the analysis of S127 phosphorylation (if possible S381/397 phosphorylation would also be interesting in this context).

RESPONSE: We have now performed Western analysis to monitor S127 and S397 phosphorylation together with S128 phosphorylation in a variety of cell lines including MCF10A, HEK293T and NIH3T3. As shown in Fig 4B of the revised version, a reduction of NLK by high density resulted in a decrease in S128 phosphorylation companying with an increase of S127 and S397 phosphorylation in all cell lines tested.

7) Extension of Figure 5:

(i) Regarding NLK mediated S128 phosphorylation of YAP and then the YAP/TEAD interaction, I think that the authors should extend Figure 5A and also determine how S128A and S128D bind to TEAD.

RESPONSE: We thank the reviewer for suggesting this helpful experiment. Because another reviewer has mentioned the quality of this data, we have used two different NLK KO pool cell lines and obtained improved data. We also carried out IP experiments with the mutant constructs indicated in Fig 5B of the revised version. YAP-S127A and -S128D, mimicking NLK-mediated phosphorylation, showed stronger interaction with TEAD than wild-type or the S127A mutant did.

(ii) Expand Figure 5G to fully link S128 phosphorylation to NLK in the context of cell migration. Does YAP S127A and S128D rescue the decreased migration of NLK knockdown cells?

RESPONSE: Yes. YAP-S127A and -S128D rescue the decreased migration of NLK knockout cells. As shown in the quantification histograms in Fig 5E of the revised manuscript, YAP-S127A and -S128D, but not YAP-S128A, rescue the decreased migration of NLK knockout cells.

8) Confirm that Yorkie target gene expression reduction upon NLK knockdown is Yorkie dependent. Is there a quick way to confirm that observed differences in Nemo deficient tissue are Yorkie dependent. For example does Hippo insensitive Yorkie rescue the expression in Nemo deficient tissue?

RESPONSE: We have now performed this experiment (Fig 5D) and found that indeed the Hippo-insensitive Yorkie form fully rescues the effect of Nemo downregulation on the target gene expression. In fact the phenotype of UAS-nmo-RNAi; UAS-yki[S168A] wing discs is indistinguishable from the discs expressing the yki[S168A] form alone, suggesting that Yorkie is epistatic to Nemo in Drosophila.

Minor points:

A) Mention other YAP S127 kinases.
Considering that it was recently reported that YAP can also be regulated by S127 phosphorylation mediated by the Ndr kinases (see Zhang et al., 2015), the authors maybe also want to mention this briefly in the discussion section. Potentially, NLK mediated phosphorylation of YAP affects more than the Lats mediated phosphorylation of YAP. - I do not expect the authors to test this experimentally, but maybe it is worth mentioning (if space allows).

RESPONSE: We have now mentioned this in the Discussion section according to the reviewer's suggestion, and the reference is now added in the revised manuscript.

B) Extend a little bit more on S127 related literature: In order to give a broader picture regarding the role of S127 phosphorylation of YAP, I think that the authors should also mention and reference the following key publication: Chen Q, Zhang N, Xie R, Wang W, Cai J, Choi KS, David KK, Huang B, Yabuta N, Nojima H, Anders RA, Pan D. Homeostatic control of Hippo signaling activity revealed by an endogenous activating mutation in YAP. Genes Dev. 2015 Jun 15;29(12):1285-97. Doi 10.1101/gad.264234.115. PubMed PMID: 26109051; PubMed Central PMCID: PMC4495399.

RESPONSE: We have now mentioned this in the Discussion section according to the reviewer's suggestion, and the reference is now added in the revised manuscript.

C) Re-phrase sentence on page 4: Please re-phrase the following: "...indicating NLK-mediated YAP phosphorylation was responsible for the mobility shift..." The authors should note that the data shown at this stage of the manuscript in the first figures do not allow them to draw this conclusion here. These experiments rather show that YAP phosphorylation can be influenced the kinase activity status of NLK.

RESPONSE: We apologize and thank you for this point. According to the reviewer's suggestion, we have corrected it into "...indicating the kinase activity of NLK was required for the mobility shift of YAP." in the revised manuscript.

D) Re-phrase sentence on page 5: Please rephrase: "To examine whether NLK directly phosphorylates Ser128 of YAP..." Again this is here a premature conclusion, since the co-expression in cells does not allow the conclusion of a direct phosphorylation. Direct phosphorylation is tested by in vitro kinase assays using recombinant proteins.

RESPONSE: We apologize and agree with this point. According to the reviewer's suggestion, we have deleted "directly" in the revised manuscript.

E) Check for NLS close to S128 phosphorylation site: could the observed localization of S128D be a consequence of influencing a NLS that is close by of the S127/S128 site?

RESPONSE: It has been well known that YAP has no conventional nuclear localization sequence. In addition, introduction of mutation into Ser127 or Ser128 did not produce any sequences similar to NLS. There is only one publication about YAP-NLS (Wang et al. (2016), Importin α1 mediates Yorkie nuclear import via an N-terminal non-canonical nuclear localization signal. J Biol Chem. 291(15):7926-37), where it is shown that the N-terminal 1-55 amino acids of Yorkie are required for its nuclear localization, but it is not functional for mammalian YAP nuclear localization. Currently, we do not know the exact mechanism of YAP nuclear localization; however, we think that the reduced interaction between YAP and 14-3-3, along with increased interaction between YAP and TEAD, both caused by the NLK-mediated YAP phosphorylation, result in increased nuclear localization of YAP.

F) Be careful with the wording on page 6: the authors do not really measure "the stability" of NLK, so I would re-phrase the text accordingly.

RESPONSE: We agree with the reviewer's point. We have corrected it into "protein level of NLK" in the text. Subheading has been also corrected as 'NLK localization and protein level are regulated in a cell density dependent manner."

G) Consider testing nuclear targeted NLK: Based on the model that is proposed on page 9, the authors maybe want to test the consequences of permanently targeting NLK to the nucleus. Not an essential point, but this would further strengthen their proposed model.

RESPONSE: It would be an interesting experiment to strengthen our model. We may investigate this issue further in cancer cells in the future. However, we wish the reviewer to understand that we have already added huge amounts of data in our revised manuscript to strengthen our findings for the EMBO Reports.

Referee #2:

The manuscript by Moon and colleagues presents an interesting and novel angle on the regulation of the transcriptional co-activator YAP. The authors suggest that NLK interacts with and directly phosphorylates YAP at Serine 128, and provide evidence that this modification promotes nuclear YAP localization and activity. Serine 128 within YAP lies adjacent to the well characterized Serine 127 that is phosphorylated in response to activation of the Hippo pathway. The authors suggest that modification of these residues act in an antagonistic manner to the other. The authors show data

supporting their model, including data that shows NLK-induced p-S128 YAP reduces phosphorylation of YAP on S127. The authors also show that a S128A mutant of YAP more strongly interacts with 14-3-3 and is localized more prominently in the cytoplasm of HeLa cells.

The authors propose an interesting new mechanism for the regulation of YAP, and identify a new post-translational modification that impacts YAP activity. However, while there is some novelty to proposed mechanism, major experimental and conceptual issues exist, particularly with respect to the relevance of NLK in the proposed mechanism. Overall, the data in the manuscript need significant improvements, and more experimental support for their proposed model in vitro and in vivo are required before publication. Some comments that need to be addressed are list below:

1) A crucial control for the kinase assay conducted in Fig 1B is testing whether purified GST is a target of NLK. This kinase assay should be conducted in parallel with GST-YAP and the kinase-dead versions of NLK.

RESPONSE: The critical point in this data is that the positive signal phosphorylated by wild-type NLK was detected at the exact molecular weight corresponding to GST-YAP. We think that using the kinase dead version of NLK is a better control for this experiment than usage of GST. We already provided the in vitro kinase assay data with the kinase-dead form (KM) of NLK. Although GST was not used in this experiment, the accompanying revised paper of Hong and colleagues used GST as a control for GST-YAP and found that GST was not phosphorylated by NLK.

Moreover, an important experiment to validate whether NLK directly phosphorylates S128 in YAP is the test whether NLK phosphorylates the S128A mutant of YAP in vitro.

RESPONSE: Based on the reviewer's suggestion, the in vitro kinase assay was performed. As shown in Fig EV3F, phosphorylation at S128 of GST-YAP, but not GST-YAP-S128A, was induced by immunoprecipitated Flag-NLK, but not NLK-KM.

2) The data in Fig 2B is not convincing to show that NLK reduces YAP phosphorylation on S127. In particular, the quantitation included should show data from at least three experiments and include error bars and statistics. Also, it is curious that NLK expression in this figure does not induce a mobility shift in endogenous YAP. Related to the prior point, why is there no mobility shift in YAP when co-expressed with NLK in Figures 2D and EV2B? This is inconsistent with the data shown in Fig 1A.

RESPONSE: We have now performed three independent experiments and present very convincing data with quantification (Fig. 2B). We can clearly see the NLK mediated mobility shift of ectopically expressed YAP when we run gels longer (at least 4h) at lower percentage of acrylamide. However, the sharpness of other bands suffered at this condition. Thus, after obtaining the first set of data (presented in Fig. 1A), we later on utilized normal SDS-PAGE conditions and relied instead on the use of phosphor-specific antibodies. The mobility shift of the ectopically expressed YAP by NLK was too obvious for us, however, we could not see a clear mobility shift of the endogenous YAP. Because the endogenous YAP might have multiple phosphorylations in different sites and the increase of S128 phosphorylation by NLK leads to a decrease in S127 phosphorylation by Lats1/2 (Fig 2B and D), we could not see the mobility shift. However, ectopically expressed YAP with NLK might be mainly phosphorylated by NLK and the mobility shift of YAP could be easily detected.

3) The figure legend for Fig EV2B seems to have an error, as it makes reference to phospho-S127 YAP quantitation.

RESPONSE: We apologize for the mistake. It is now corrected.

4) The authors make the statement that their data "suggest that LiCl-mediated induction of YAP phosphorylation of Ser127 was due to inhibition of NLK". LiCl may inhibit many kinases beyond GSK3b and therefore a conclusion about NLK from this experiment cannot be made. This statement needs to be revised, and if this data is included, a way of better linking this experiment to NLK should be considered. For example, can LiCl inhibit the effects of NLK overexpression? Can LiCl affect p-S128 YAP levels?

RESPONSE: Based on the reviewer's suggestion, we have now performed an experiment to test whether NLK mediated phosphorylation of Ser128 can be blocked by the treatment with LiCl. Yes. LiCl inhibited the effect of NLK overexpression (Fig EV3E).

5) The knockdown of GSK3b in Fig EV3B is very minimal. Based on this data, the authors cannot make any statements about whether GSK3b affects phosphorylation of YAP on S127. As alluded to in the prior point, using LiCl as a means to assess NLK activity is not ideal and I would suggest using alternative methods to inhibit NLK activity.

RESPONSE: We used shGSK3b in the previous version of the manuscript. To improve the knockdown efficiency, we used siRNA for GSK3b for the revision experiments and were able to get convincing data (Fig EV3B). While the knockdown of GSK3b did not change the level of pYAP-S127 (Fig EV3B), the level of pYAP-S127 was significantly enhanced in two independent NLK knockout pool cell lines (Fig 2C).

6) The quantitation in Fig 2C should show data from at least three experiments and include statistics.

RESPONSE: Because the knockdown efficiencies were varied in different experiments, we have now used the CRSPR-Cas9 system to get better and more reliable results. We generated two different NLK KO pool cell lines. We have performed three independent experiments with these KO cell lines including the pYAP-S397 readout (as suggested by reviewer #1) and showed quantification in the right panel of Fig 2C. The data obtained using NLK KO cell lines is similar to the results shown in previous Fig 2C with NLK siRNA.

7) The data in Fig 2E is not convincing for the conclusions made by the authors. The total levels of WT and S128A YAP are not equivalent and the increased levels of S128A YAP may explain why there is an observed higher level of p-YAP(S127). Also, it is difficult to make a strong conclusion about p-YAP(S128) given the non-specific band. The authors do not seem to observe a similar non-specific band in Fig EV2B, so it seems possible to improve this data.

RESPONSE: Improved data are now presented as Fig 2E in the revised manuscript.

8) While the authors show that the pS128 antibody recognizes overexpressed wild type YAP, but not S128A-YAP, the authors do not provide any controls showing that the antibody recognizes endogenous YAP. The authors should include a knockdown experiment of YAP showing that this antibody is specific for endogenous YAP.

RESPONSE: We have not tried to knockdown YAP, however, as shown in Fig 4B, the level of pYAP(S128) is reduced along with the decrease in the level of YAP and NLK in high cell density, which suggests that pS128 antibody is specific for the endogenous YAP. In addition, we have added data for endogenous levels of pYAP-S128 after overexpression of NLK (WT or KM) and the KO of NLK, as shown in Fig EV3D and Fig 5A, respectively.

9) It is unclear why reference is made to Flag-NLK in the Fig 3B image.

RESPONSE: Flag-NLK should have been labelled as Flag-YAP in Fig 3B of the original version. We apologize for this mistake, which is now fixed (Fig 3C). Because we think that the reviewer's concern is due to our labeling mistake, we provide below (but not in the main text) an additional explanation of this experimental setup. Our aim was to test whether the NLK-mediated phosphorylation status of YAP on Ser128 affected the interaction between YAP and 14-3-3. To test whether different phosphorylations on YAP provide differential ability to interact with 14-3-3, we did the IP experiment with different YAP phosphor mutant forms, and found that YAP-S128A showed a stronger interaction with 14-3-3 than YAP-WT did, whereas neither YAP-S127A nor YAP-S128D showed binding to 14-3-3.

10) It is unclear why the authors examined the localization of GFP-tagged YAP in Fig EV4. The authors should test whether NLK expression induces endogenous nuclear Yap localization. Also, all experiments showing YAP localization changes should include quantitation, as a small field of view may not represent the overall changes.

RESPONSE: Regarding this concern, reviewer 3 suggested the CRISPR/Cas9 system to get better and more reliable results. We have generated two different NLK KO pool cell lines. As shown in Fig 3E of the revised manuscript, our results clearly show that cells depleted of NLK display cytosolic localization of YAP at low density. We think that it is not necessary to quantify for YAP localization changes because large fields of view are now shown.

11) The quantitation in Fig 4B should show data from at least three experiments and include error bars. Also, the levels of pS127-YAP and p-S128-YAP should be shown in this experiment.

RESPONSE: To better resolve this concern, we decided to test it in three different cell lines: MCF10A, HEK293T and NIH3T3. These results not only reveal the reproducibility of this effect, but they also provide a cell-context independent readout. In addition, as the reviewer has suggested, we tested the levels of pYAP-S127 and pYAP-S128 in same cell lysates. Consistent with the reduction of the NLK levels at high cell density (HD), pYAP-S128 was decreased while pYAP-S127 was up-regulated. The new data has been added in Fig 4B of the revised version.

12) An IgG control needs to be included in the IP experiment shown in Fig 4C to rule out non-specific bands, particularly given that the p-S128 antibody is shown to have non-specific bands associated with it.

RESPONSE: When we prepared the first version of the manuscript, we were not able to detect the endogenous pYAP-S128 without immunoprecipitation of YAP. However, further purification of the pYAP-S128 antibody during the preparation of revision has enabled us to detect endogenous pYAP-S128. Therefore, we think that the previous Fig 4C is not necessary, and replaced it with the new version of Fig 4B.

13) It is interesting that NLK exhibits similar changes in localization as seen with YAP upon cell density changes. However, it is unclear what this data means in the context of NLK function. Why is this important? Does NLK interact with YAP only in the nucleus? If so, what is the significance of this occurring under low density conditions? For example, is p-S128 modification of YAP important for its cell proliferation roles, or it's transcriptional activity? How is NLK cytoplasmic localization induced upon cell density, and why are NLK levels reduced? Is NLK regulated by the Hippo pathway? Without some kind of mechanistic insight into how density affects NLK activity on YAP, Fig 4 does not add any information that is related to YAP.

RESPONSE: We appreciate the reviewer's thoughtful comments. The suggested experiments would be a truly excellent project for a new graduate student and a new grant proposal. However, we think that it would be far beyond the scope of the current manuscript.

14) An IgG control needs to be included in the IP experiment shown in Fig 5A to rule out non-specific bands. This is very important for detecting proteins such as TEAD1, as the protein runs almost exactly with the heavy chain of the antibody used for the IP. Also, the levels of pS128-YAP should be included in the analysis of the lysates.

RESPONSE: We thank the reviewer for suggesting this helpful experiment. Because another reviewer also mentioned the quality of this Figure, we have used two different NLK KO pool cell lines and obtained improved data including the level of pYAP-S128 (Fig 5A of the revised manuscript). We also carried out immunoprecipitation experiments with the mutant constructs as indicated in Fig 5B of the revised version. S127A and S128D that mimic NLK-mediated phosphorylation showed stronger interaction with TEAD than wild-type or S128A mutant did.

15) The authors should correct the y-axis in Fig 5C as the data is from a qPCR experiment.

RESPONSE: We apologize and have corrected this. This Figure is now moved to Fig EV6A of the revised manuscript.

16) The effects of NLK knockdown on expression changes of YAP target genes was assessed only in HEK-293T cells. It would strengthen the paper to show data from a similar experiment in another cell line.

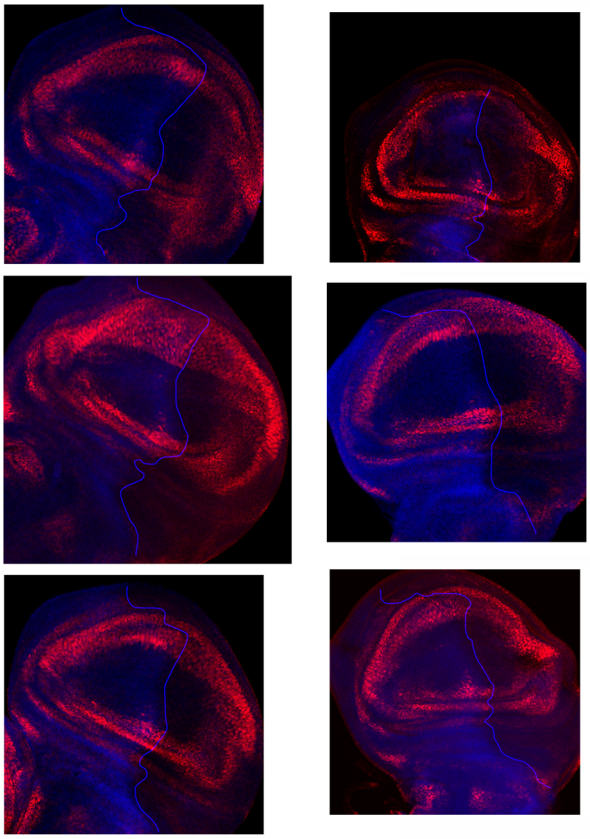
RESPONSE: We examined other YAP targets in Drosophila (Fig 5C and Fig EV6C) instead of assessing the changes of in YAP target genes in another cell line.

17) Many of the experiments throughout the paper use different cell lines, switching between mouse and human and epithelial and fibroblast. No justification for switching cell lines for the different experiments is given. Also, no information on whether NLK is expressed and to what level it is within these different cell lines is given. The use of each cell line and the rationale should be included in the manuscript.

RESPONSE: Throughout our manuscript we mainly used HEK293 cells for the transient transfection experiments since the transfection efficiency of these cells is high. Other cell lines used by us are also routinely used in the Hippo signaling field and it is difficult to provide specific justification. We have examined NLK levels in different cell lines (Fig 4B). More importantly, consistent findings in different cell lines strongly support the idea that our model is applicable in a cell context-independent manner (Fig 3D and E, Fig 4, and Fig EV4 and 5).

18) The data in Fig 5D is not convincing. Based on the image shown, it does not appear that nmo-RNAi leads to reduced expanded levels. Similarly, the images in Fig 5E are also not convincing. Moreover, to draw conclusions from these in vivo experiments Nemo levels need to be verified that they are being knocked down or induced.

RESPONSE: The reviewer raises several points here, which we wish to address one by one.



(Revised version of Fig 5C)

First, the reviewer is not convinced by the images on exLacZ levels upon nemo RNAi expression. We now stress in the text that normal expression of exLacZ is low within the wing pouch and high at the wing pouch periphery (see e.g. Djiane et al, PLoS ONE 2014). This is why to test if nemo RNAi leads to a reduction in exLacZ levels, we must concentrate on the pouch periphery region, which we do, finding a clear reduction in exLacZ levels within the posterior, RNAi-expressing zone, as compared to the wild-type anterior zone (Fig EV6C of the revised manuscript). For additional illustration, we here provide immunostaining of six additional discs of the genotype of the panel EV 6D (panel 5D of the initial submission).

In addition to these clarifications, we have now provided analysis of the effect of nemo downregulation on expression of another Yorkie target gene DIAP1, more uniformly expressed throughout the wing pouch, and find a clear reduction in DIAP1 levels upon nemo downregulation

Second, the reviewer is not convinced that nemo overexpression results in upregulation of the Yorkie target gene expression. Although we think the image presented is very illustrative, we have now added quantification of this target gene expression upon nemo overexpression (Fig EV6C or revised manuscript), which reveals a strong and unequivocally significant effect.

Third, the reviewer asks for the verification of Nemo up- and down-regulations by antibody staining. Unfortunately, despite years of research on Drosophila Nemo and numerous publications by several laboratories, none has succeeded in producing anti-Nemo antibodies acting in immunostaining.

Within the time frame given for the revision, we did not even dare to produce such antibodies, given the fact that everybody else has failed. However, we have made a trial with polyclonal antibodies form Aviva, which have been raised against a conserved peptide within the core of NLK and are reported to recognize NLK in species from humans down to zebrafish. As this peptide is conserved in Drosophila nemo too (with some changes though), we hoped that this antibody might eventually recognize the Drosophila nemo. Alas, they did not. Thus, our only response to this important point of the reviewer is that for the overexpression of nemo, we used the line which has been previously published and well-characterized (Verheyen et al., Mech Dev 2001). As for the nemo downregulation, as explained in the figure legend and Methods, two different UAS-RNAi lines have been used by us with similar outcome.

19) Antibodies exist for examining Yorkie phosphorylation in Drosophila, and therefore to validate the mechanism proposed by the authors in this model system the authors should show that alterations in Nemo levels affects Yorkie phosphorylation. The authors should also test whether the conserved S128 residue regulated Yorkie in the same way as it does in YAP. If the p-S128 YAP antibody recognizes Yorkie, it could be used to experimentally to examine whether Nemo regulates Yorkie.

RESPONSE: We appreciate the editor for excluding this concern. Although these are important questions, addressing them would have taken more time than allocated for the revision. Thus, given the permission of the editor, we decided not to address these issues.

20) It is unclear from the cell migration assays in Fig 5F how this relates to YAP. While it is interesting that NLK knockdown reduces cell migration, there are a multitude of ways this could be achieved and therefore no relationship to YAP, or phosphorylation of YAP, can be drawn from this data.

RESPONSE: We thank the reviewer for this opportunity to improve our findings. As shown in Fig 5E of the revised manuscript, cell migration reduced by NLK knockdown was rescued when YAP was expressed. Thus, together with other results, these data suggest that NLK acts at the upstream of YAP.

21) More details for how relative width was calculated from area measurements in Fig 5G should be included. Importantly, for these experiments, parallel analyses of the levels of the various YAP mutants needs to be determined so that any differences can be attributed to the mutation rather than the differences in expression.

RESPONSE: We used TScratch software which can be downloaded free of charge, and cited the reference (Geback et al. (2009) TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays. Biotechniques 46: 265-74). Based on reviewer's suggestion we added following sentence in the Figure legend. "Graphs show percentage of unfilled areas at 24 hr compared to 0 hr, and average values from a representative of multiple experiments performed in triplicate." In addition, we changed the way of presenting this result, which is more straightforward to understand. To resolve the reviewer's concern, we tested wild-type and phosphor mutant form of YAP in NLK KO cell lines. Consistent with other data, YAP-S127A and -S128D rescued the decreased migration of NLK knockout cells. As shown in the quantification histograms in Fig 5E of the revised manuscript, YAP-S127A and -S128D, but not YAP-S128A, rescued the decreased migration of NLK knockout cells. Also, we showed similar expression of wild-type and mutants of YAP by immunoblotting (Fig 5E).

Referee #3:

Moon et al., report NLK as a kinase that phosphorylates Yap and activates it. Their data lend some support to a mechanism whereby NLK-mediated S128 phosphorylation activates YAP by preventing LATS1/2-mediated phosphorylation of S127. The studies uses biochemistry, human cultured cell assays and some fly genetics to make a reasonably convincing case. The physiological context in which this phosphorylation occurs is not explored. The manuscript has promise but many things need substantial attention, including some key experiments that address how important NLK regulation of YAP is. Further, the manuscript relies mostly on overexpression tissue culture studies.

Loss of function studies are crucial. The conclusions don't match the data at present and more detailed analysis of endogenous NLK is essential.

1) In Figure 1C the NEK/YAP is not overly convincing. Have these been proteins ben found to interact in the many unbiased proteomic studies that have been performed with YAP?

RESPONSE: NLK has not been found in unbiased proteomic studies that aimed to identify interaction partners of Hippo signaling. However, there are many examples that identified novel binding partners were unrecognized in proteomic studies. For example, Setd7 is a SET-domain-containing lysine methyltransferase and binds Yap to promote its cytoplasmic sequestration (Oudhoff et al., Dev Cell., 2013). Yet Setd7 has not been mentioned as a binding partner by the many unbiased proteomic approaches. Also, the poly-ADP-ribosylating enzyme tankyrase binds Axin, a negative regulator of Wnt signaling pathway, to stimulate its degradation, but it was also never identified in any of the proteomic approaches. We expect that it is difficult to identify enzymes such as NLK using proteomic studies because they easily dissociate from its substrate after modifying its target. Actually, we also showed that the kinase-dead form of NLK has better binding affinity to YAP than the wild-type kinase. By the way, we repeated the immunoprecipitation experiments with either anti-YAP antibody or anti-NLK antibody and now present more convincing data (Fig 1C).

2) Figure 2- Does the P-S128-YAP antibody detect endogenous P-S128-YAP? Two important experiments are missing that relate to the importance of NLK-mediated phosphorylation of YAP:
1. Does depletion or CRISPR-mutation of NEK reduce/ablate P-S128-YAP phosphorylation?

RESPONSE: Thank you for suggesting the NLK knockout experiment using the CRSPR-Cas9 system. Using the NLK knockout cells has allowed us to get clearer and more consistent data than using the siRNA-mediated NLK knockdown cells. As shown in Fig 5A, the level of pYAP-S128 was reduced in NLK knockout cells. In addition, ectopic expression of wild type NLK, but not the kinase activity dead mutant form of NLK, enhanced the level of pYAP-S128 (Fig EV3D).

2) Does depletion or CRISPR-mutation of NEK increase P-S127-YAP phosphorylation by LATS1/2? These sorts of experiments are important to determine how important NEK is for YAP regulation. SO far this is not addressed at all in the manuscript.

RESPONSE: As described above, we have generated two different NLK KO pool cell lines using the CRSPR/Cas9 system. The NLK KO pool cell lines clearly show increased levels of pYAP-Ser127 and pYAP-Ser397 which are well-known phosphorylation sites by Lats1/2 (Fig 2C and 5A).

3) Figure 3 - Does NLK LOF increase YAP/14-3-3 binding and YAP localization in cells?

RESPONSE: We appreciated this, which permitted us to improve our findings and conclusions. Using two different NLK KO cell lines, we carried out the IP experiment to test interaction between endogenous YAP and 14-3-3 and examined YAP localization. We observed consistent results that loss of NLK led to increased interaction between endogenous YAP and 14-3-3 (Fig 3B). Also, NLK KO pool cells clearly exhibited a robust cytoplasmic localization of endogenous YAP when compared with wild-type cell lines (Fig 3E).

4) Figure 4 - 4C is a key result. It should be quantified from multiple experiments and more of the P-YAP-S128 blot should be shown. Why do you think there are multiple bands here?

RESPONSE: When we prepared for first version of manuscript, we were not able to detect endogenous pYAP-S128 without immunoprecipitation of YAP. However, further purification of the pYAP-S128 antibody during preparation of the revision enabled us to detect endogenous pYAP-S128. To better resolve this concern of the reviewer, we decided to apply three different cell lines: MCF10A, HEK293T and NIH3T3. The results we obtained not only reveal reproducibility of the finding, but further demonstrate it in the cell-context independent manner. In addition, as the reviewer has suggested, we tested the levels of pYAP-S127 and pYAP-S128 in the same cell lysates. Consistent with the reduction of NLK levels at high cell density (HD), pYAP-S128 was decreased while pYAP-S127 was up-regulated. The new data without nonspecific bands has been added in Fig 4B of the revised manuscript.

5) Can NLK siRNA increase YAP-14-3-3 interaction in low-density cells?

RESPONSE: This comment is very similar to the point #3 raised by the reviewer. Please note our response for point #3.

6) Figure 5 - the IPs in 5A are not convincing. Show more of the blots.

RESPONSE: We thank the reviewer for suggesting this experiment. Because other reviewers also want us to make it more convincing, we have used two different NLK KO pool cell lines and obtained improved data (Fig 5A of the revised version). We also carried out IP experiments with the mutant construct as indicated in Fig 5B of the revised version. YAP-S127A and -S128D that mimic NLK-mediated phosphorylation showed stronger interaction with TEAD than wild-type or YAP-S128A mutant did.

7) The wound assays in 5G need to be repeated and quantified. Does S128A close the wound faster than WT? If not, then what is the importance of NLK? AT preswnt hteis claim is unfounded without quantification: "Wound healing ability was affected by the phosphorylation status of Ser127 or Ser128."

RESPONSE: We thank the reviewer for bringing this up. Similar concerns have been raised by the Reviewer 1 and 2. To resolve this concern, we tested the wild-type and the phosphor mutant forms of YAP in the NLK KO cell lines. Consistent with the other data, YAP-S127A and -S128D rescued the decreased migration of NLK knockout cells. As shown in the quantification histograms in Fig 5E of the revised manuscript, YAP-S127A and -S128D, but not YAP-S128A, rescued the decreased migration of the NLK knockout cells. Also, we showed similar expression of the wild-type and mutants of YAP by immunoblotting (Fig 5E).

8) The data in Drosophila tissues are important because without in vivo experiments the impact of this mostly cell culture based study is far less. However these studies are presently weak and require substantial improvement to be convincing. The current expanded-lacZ stainings seem to skim the top of the disc and need to come from the disc proper. It is quite possible that Nemo modulation causes a morphological change to the disc and that is what we see in Fig 6, rather than a change in expanded-lacZ levels.

The authors should do the following:

i) Capture images that are clearly from the middle plane of the disc, rather than the apical or basal surface, ii) Capture an X-Z section, and iii) also image one or more independent readouts of Hippo pathway activity, such as bantam or DIAP1, to validate the expanded-lacZ experiments.

RESPONSE: The reviewer raises several issues here, which we will address one by one.
First, the reviewer is not convinced by the exLacZ staining upon nemo downregulation. We now stress in the text that normal expression of exLacZ is low within the wing pouch and high at the wing pouch periphery (see e.g. Djiane et al, PLoS ONE 2014). This is why to test if nemo RNAi leads to a reduction in exLacZ levels, we must concentrate on the pouch periphery region, which we do, finding a clear reduction in exLacZ levels within the posterior, RNAi-expressing zone, as compared to the wild-type anterior zone (Fig EV6D; also see an additional figure provided above in our response to the reviewer 2 questions). In addition to these clarifications, we have now provided analysis of the effect of nemo downregulation on expression of another Yorkie target gene DIAP1, more uniformly expressed throughout the wing pouch, and find a clear reduction in DIAP1 levels upon nemo downregulation (Fig 5C). We also assure the reviewer that the images were taken indeed from the middle plane of the disc. Second, to continue along these lines, the reviewer asks for a Z-stack, which we now provide in Fig 5C. Third, the reviewer asks to add another Yorkie target gene, like bantam or DIAP1, to validate the expanded-lacZ experiments. According to this suggestion, we have now added DIAP1 analysis.

9) What about epistasis experiments? These could really help to strengthen the manuscript.

RESPONSE: As the editor suggested to us that fully addressing this important issue was not obligatory (given the fact that this would have taken more time than allocated to us for revision), we did not elaborate extensively on this issue during revision. However, we do provide some epistasis analysis (Fig 5D and respective parts of the text), which suggests that Yorkie indeed acts downstream from Nemo, as expected by our model.

10) The authors should far more of some Western blots - e.g. Fig 4C, 5A and 1C.

RESPONSE: We have added more data to make them convincing (Fig 1C, Fig 4B, Fig 5A and B).

2nd Editorial Decision 07 October 2016

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the three referee reports that you will find enclosed below. As you will see, all three referees support the publication of your manuscript in EMBO reports. However, referee #2 raises some further concerns that we ask you to address during a final revision.

Further, I have a few editorial requests.

The title is currently too long. Please provide a shorter title with less than 100 characters. Also the abstract need s to be shortened to 175 words (or less).

For a short report in EMBO reports, we require that the results and discussion sections are combined in a single section called "Results and Discussion". Please do that.

The scale bars in the microscopic images differ in thickness from panel to panel. Sometimes, the scale bars will be hardly visible in the final online version (e.g. Fig. 3E or EV6E). Please provide clear to see scale bars of similar thickness for all microscopic panels. Please also add scale bars to Fig. EV6C/D.

Regarding data quantification and statistics, can you please specify the number "n" for how many experiments were performed and the test used to calculate p-values in ALL the respective figure legends? This information must also be provided in short paragraph in the methods part (statistical analysis).

Finally, currently you have 6 EV figures. Generally, we allow only 5 EV figures for a short report. If possible, please fuse two of the current EV figures (e.g. EV5 and EV6) to have 5 EV figures in the final revised version. Please update the call-outs in the manuscript text and the figure legends accordingly.

REFEREE REPORTS

Referee #1:

All my major points have been addressed sufficiently.
1) It is a pity that the anti-S128-P and anti-S397-P antibodies are not 100% specific (at least when tested on bacterially expressed recombinant YAP). Nevertheless, I think that the authors have sufficiently addressed this point, in particular with showing that S128D is weaker phosphorylated on S127 by Lats1. The S397 phosphorylation was also addressed sufficiently in cells.
2) Sufficiently addressed.
3) It would have been nice to see an extension of Figure 1B, but given all the other evidence provide in this manuscript, I think that the written response by the authors is sufficient.
4) Sufficiently addressed by new experiments or scientific argumentation.
5) Sufficiently addressed by new experiments or scientific argumentation.
6) Sufficiently addressed by new experiments or scientific argumentation.
7) Sufficiently addressed.
8) Sufficiently addressed.

All my minor points have been addressed sufficiently.

Referee #2:

The revised manuscript by Moon et al has made significant improvement, but there are still some issues that were brought up in the first round of reviews that were not addressed. Specifically, I have two issues regarding experimental controls that should be addressed before the manuscript is suitable for publication:

1. I strongly feel that the GST control for the NLK-YAP kinase assay should be included in the manuscript. GST is well known to be promiscuously phosphorylated in vitro by many kinases, and it is possible that GST is phosphorylated by NLK in the experiments presented in the manuscript (i.e. the GST fused to YAP - which would run at the position of GST-YAP). This GST control needs to be included in parallel with analysis of GST-YAP phosphorylation so that the kinase activity can be compared with the same experimental setup. Without this control the authors cannot claim that NLK phosphorylates YAP in vitro. Also, it is unacceptable to refer to non-reviewed/published communication of experiments performed by another group to try argue against performing a critical control.

2. It seems very simple to show that the pYAP-S128 antibody specifically recognizes endogenous YAP by knocking down YAP and examining lysates by western blotting, so it is unclear why this control was not included in the revised manuscript. Given that this is a new antibody, and this antibody is used for making strong conclusions (including conclusions on endogenous YAP), I would like to see this control.

Additional minor comment:

While it does appear that nmo-RNAi expression leads to reduced DIAP1 levels in the Drosophila wing discs shown, the relationship is not clean across the A-P border. For example, there are clear regions near the border that show high levels of DIAP1 that are unaffected by nmo-RNAi. This should be pointed out in the text, and a comment should be made somewhere in the manuscript about the potential non-autonomous effects of nearby cells or lack of Nmo expression in some cells at the clone borders.

Referee #3:

The authors have attempted many experiments to respond to reviewer critique and the manuscript has been improved.

2nd Revision - authors' response 11 October 2016

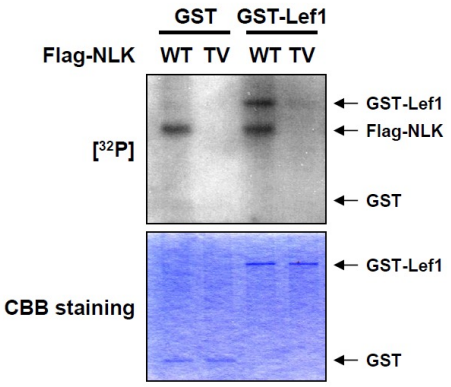
Referee #2 (Report for Author)

The revised manuscript by Moon et al has made significant improvement, but there are still some issues that were brought up in the first round of reviews that were not addressed. Specifically, I have two issues regarding experimental controls that should be addressed before the manuscript is suitable for publication:

1. I strongly feel that the GST control for the NLK-YAP kinase assay should be included in the manuscript. GST is well known to be promiscuously phosphorylated in vitro by many kinases, and it is possible that GST is phosphorylated by NLK in the experiments presented in the manuscript (i.e. the GST fused to YAP - which would run at the position of GST-YAP). This GST control needs to be included in parallel with analysis of GST-YAP phosphorylation so that the kinase activity can be compared with the same experimental setup. Without this control the authors cannot claim that NLK phosphorylates YAP in vitro. Also, it is unacceptable to refer to non-reviewed/published

communication of experiments performed by another group to try argue against performing a critical control.

Response: We apologize not to include GST control for NLK-YAP kinase assay in the revised manuscript. GST can be phosphorylated by several kinases as raised by the reviewer. However, our data shows that GST is not phosphorylated by NLK performed as same condition for our NLK-YAP kinase assay. As shown below, GST-Lef1, widely used as a positive control for NLK activity (Ishitani et al. (1999). The TAK1-NLK-MAPK-related pathway antagonizes signalling between beta-catenin and transcription factor TCF. Nature 399: 798-802), is efficiently phosphorylated by NLK, while GST was not. Thus, although we did not include the control in revised manuscript, our data suggest that NLK does not phosphorylate GST in vitro.



2. It seems very simple to show that the pYAP-S128 antibody specifically recognizes endogenous YAP by knocking down YAP and examining lysates by western blotting, so it is unclear why this control was not included in the revised manuscript. Given that this is a new antibody, and this antibody is used for making strong conclusions (including conclusions on endogenous YAP), I would like to see this control.

Response: We thought that showing the reduced level of pYAP-S128 by knocking down YAP is not a good way to prove the specificity of pYAP-S128 antibody. It will simply show that pYAP-S128 antibody detects YAP. We think that newly added experimental data in the revised manuscript is a better way to prove specificity of pYAP-S128 antibody. Followings are the supporting pieces of evidence.

[Major pieces of evidence]

1. *Fig EV3C: The in vitro kinase assay shows that no positive signal was detected upon expression of YAP-S128A unlike wild-type YAP by immunoprecipitated Flag-NLK.*
2. *Fig EV3D and Fig 2D: Ectopic expression of wild type NLK, but not kinase-negative NLK, enhanced the level of endogenous and exogenous phosphor-YAP-Ser128.*

[Other pieces of supporting evidence]

1. *Fig 5A: YAP-S128 phosphorylation was reduced in NLK KO cell pool.*
2. *Fig 2E: pYAP-S128 antibody did not detect EGFP-YAP-S128A.*
3. *Fig EV3E: LiCl, an inhibitor of NLK, efficiently blocked phosphorylation of EGFP-YAP-S128 induced by Flag-NLK.*

Additional minor comment:

While it does appear that nmo-RNAi expression leads to reduced DIAP1 levels in the Drosophila wing discs shown, the relationship is not clean across the A-P border. For example, there are clear regions near the border that show high levels of DIAP1 that are unaffected by nmo-RNAi. This should be pointed out in the text, and a comment should be made somewhere in the manuscript

about the potential non-autonomous effects of nearby cells or lack of Nmo expression in some cells at the clone borders.

The feature, which attracted attention of the reviewer, stems from the fact that DIAP1 expression in Drosophila wing discs, as described by many publications, is of a salt-and-pepper type. In other words, there naturally exists a certain levels of cell-to-cell variability in DIAP1 expression, be it in the normal wing discs (or the unaffected anterior region of the wing discs shown in Fig. 5C), or in the posterior regions of the discs where Nemo is downregulated. This is why some cells even within the nemo-RNAi region may show relatively high levels of DIAP1. What is important is that the overall level of DIAP1 is strongly reduced within the nemo-RNAi domain, as is clearly seen in the presented image and as is confirmed by the quantification analysis over many discs.

We have now modified the sentence on p.8 of the paper to reflect this fact as follows: "To test whether Nemo downregulation could suppress another Yorkie target gene, whose expression would be more uniform across the wing pouch area, we performed immunostaining against the Yorkie target DIAP1, which reveals a salt-and-pepper expression pattern within the wing pouch area". To further support this statement, we put an additional reference at the end of this sentence (Yoo et al, Nat Cell Biol 2002).

3rd Editorial Decision - Acceptance 13 October 2016

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.

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: Vladimir L. Katanaev and Eek-hoon Jho
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-42683V1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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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?	NA
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2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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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).	appropriate citations are provided in the text
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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D- Animal Models

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9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

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
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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 accession codes for deposited data. See author guidelines, under '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	NA
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
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G- Dual use research of concern

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