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Osmotic stress-induced phosphorylation by NLK at Ser 128 activates YAP

Audrey W. Hong, Zhipeng Meng, Hai-Xin Yuan, Steven W. Plouffe, Sungho Moon, Wantae Kim, Eek-hoon Jho, and Kun-Liang Guan

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. 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 2 (actin changes), 9 (other residues than S128), 11 (CRISPR/Cas9) and 13 (YAP targets) of referee #2 and point 2 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:

Hong et al. report in this very interesting manuscript several points that are important for the broader Hippo signalling community. This manuscript is well-presented and the overall implications of the research findings have great potential. However, I think that this manuscript can benefit from some adjustments/extensions as outlined below. I firmly believe that by addressing these points this manuscript will be even more appreciated by the Hippo signalling community and the scientific community at large.

Main points:

1) Correct referencing on page 4:

On page 4 the authors state that: "... which is known to promote LATS kinase activity [28]". I am very familiar with the review that is cited as reference 28, so I think that is quite inadequate to be cited in this context. Therefore, I strongly suggest to rather cite instead original publications such as:

Praskova M, Xia F, Avruch J. MOBKL1A/MOBKL1B phosphorylation by MST1 and MST2 inhibits cell proliferation. Curr Biol. 2008 Mar 11;18(5):311-21. doi:10.1016/j.cub.2008.02.006. PubMed PMID: 18328708; PubMed Central PMCID: PMC4682548 and Hoa L, Kulaberoglu Y, Gundogdu R, Cook D, Mavis M, Gomez M, Gomez V, Hergovich A. The characterisation of LATS2 kinase regulation in Hippo-YAP signalling. Cell Signal. 2016 May;28(5):488-97. doi: 10.1016/j.cellsig.2016.02.012. Epub 2016 Feb 18. PubMed PMID: 26898830.

2) Include statistical analyses to support all "significant" statements. The authors repeatedly state that they observed "significant" changes, which consequently should be backed up by the statistical evaluation of at least three independent experiments. Fortunately, this should be rather easy for Moon et al. to address, since they state in the Methods section (in the subsection "Statistical analysis") that: "Each experiment was repeated three times..." Therefore, please include a statistical analysis of the three independent experiments for:
(i) Figure 1C (change of Lats1 kinase activity)
(ii) Figure 2E (change of 14-3-3/YAP interaction)
(iii) Figure 4D (S128-P in NLK null vs. wild-type cells)
(iv) Figure 4F (change of 14-3-3/YAP interaction) - to match/complement Figure 2E

3) Expand IF studies by biochemical fractionation experiments: In order to further support their IF studies the authors should include the WB analysis of nuclear/cytoplasmic fractionations for the following figures:
(i) Figure 1D - this is very important and should also include the analysis of S127-P
(ii) Figure 2B - to complement the analysis of Figure 1D
(iii) Figure 3B - to fully establish the importance of NLK
(iv) Figure 4H - it would be really important to back up the IF pictures, to fully establish that S128D is a mainly nuclear form irrespective of the conditions.

4) Explain the result shown in Fig 4B: According to Fig 4A, the anti-Ser128-P antibody is specific. However, in Fig 4B recombinant YAP is detected by this antibody. How do the authors explain this? Is the antibody not as specific as hoped? Or is Ser128 phosphorylated in bacteria? Or...?

5) Define the Ser127 phosphorylation status of S128A and S128D tested in Figure 4. Considering the very striking IF results shown in Figure 4H, it would be important to also define the Ser127 phosphorylation status of the S128A and S128D mutants of YAP by immunoblotting. Does S127-P negatively correlate with the nuclear localisation of S128D?

6) Re-label and expand Figure 5: Considering that Figure 5 is the main figure regarding the

biological relevance of the Ser128 phosphorylation of YAP, I think that this figure needs to be improved.

(i) First of all, all three experiments needed to be presented as n=3 with a corresponding statistical analysis of differences.
(ii) The labelling in Figure 5B needs to be adjusted. Instead of "apoptotic" it should state "cell death" or "sub-G1", since this analysis does not allow any conclusion regarding the nature of the cell death as currently is implicated by the labelling.
(iii) And most importantly (and quite essential), the experiments shown in Figure 5 that are comparing cells expressing YAP WT, S128A or S128D need to be compared to their proper control, which are empty vector expressing cells. - How much proliferation decrease/cell death/apoptosis is altered in cells expressing empty vector alone without any overexpression of any YAP variant?

7) Question regarding the in vitro kinase assay (as described in the Methods section): Why did the authors use mild lysis buffer conditions to measure the activities of Lats1 and NLK? Would it not be better to use stringent buffer conditions to be sure that you are only measuring the kinase at hand and not any potentially co-immunoprecipitating kinase (that it more likely to co-complex in mild conditions than under stringent conditions)?

Minor points:

A) Please add page numbers.
B) Add references/text in introduction section: In order to give the non-expert reader a little bit more information in the introduction section, Hong et al. should:
(i) 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.
(ii) Include the two following key publications in the context of the regulation of Lats1/2 by Mst1/2:
Praskova M, Xia F, Avruch J. MOBKL1A/MOBKL1B phosphorylation by MST1 and MST2 inhibits cell proliferation. Curr Biol. 2008 Mar 11;18(5):311-21. doi:10.1016/j.cub.2008.02.006. PubMed PMID: 18328708; PubMed Central PMCID: PMC4682548.
Hoa L, Kulaberoglu Y, Gundogdu R, Cook D, Mavis M, Gomez M, Gomez V, Hergovich A. The characterisation of LATS2 kinase regulation in Hippo-YAP signalling. Cell Signal. 2016 May;28(5):488-97. doi: 10.1016/j.cellsig.2016.02.012. Epub 2016 Feb 18. PubMed PMID: 26898830.
(iii) Describe briefly the S127 and S381 (S397) phosphorylation of YAP as regulatory mechanisms (including some key references).

Referee #2:

The manuscript by Hong et al outlines a mechanism by which osmotic stress, which can be induced by the introduction of high levels of Sorbitol or NaCl in cell growth media, induces the nuclear localization and activity of the Hippo pathway effectors YAP and TAZ. The authors describe a mechanism by which phosphorylation of YAP on Ser128 upon osmotic stress increases nuclear YAP localization and activity. Ser128 lies adjacent to the well characterized Ser127 within YAP, which is phosphorylated in response to Hippo pathway activity and controls YAP binding to 14-3-3, which is a major mechanism thought to induce YAP cytoplasmic restriction. The authors propose that phosphorylation of S128 disrupts 14-3-3 binding and thereby increase nuclear YAP. The authors also suggest that NLK is the kinase that mediates YAP-S128 phosphorylation in response to osmotic stress, and provide evidence that mutation of Ser128 within YAP leads to increased nuclear YAP activity. The manuscript outlines a new biological mechanism for YAP/TAZ regulation and identifies a novel kinase that controls YAP activity, which together make this manuscript conceptually interesting. However, several things need to be addressed before the data is suitable for publication, most of which are outlined below:

1) - The proposed mechanism of osmotic stress regulating YAP/TAZ phosphorylation/activity is inconsistent with the data published by the same group. A previous publication from the Guan Lab (Mo et al, 2015) Supplemental Figure S1 shows 25mM and 50mM Sorbitol shows no changes in YAP phosphorylation, which contradicts the data presented in Fig1A. What is the explanation for this? Can the authors demonstrate at what concentration (below 0.2M used in this submission) of Sorbitol alters the phosphorylation of Yap? Why would altered levels of osmotic stress impact YAP differentially?

2) - Osmotic stress has long been known to impact actin cytoskeleton remodeling, and therefore given the relationship between actin regulation and YAP/TAZ localization it is possible that changes in the actin cytoskeleton mediate the observed changes described by the authors. The authors should determine how the concentrations of Sorbitol and NaCl impact the actin cytoskeleton in the cells that they are using, and determine whether these potential changes impact YAP/TAZ. It would also be very interesting to examine whether NLK-mediated phosphorylation of YAP/TAZ is regulated by actin changes.

3) - Quantitation of all the presented immunofluorescence experiments need to be included.

4) - The authors should show by biochemical fractionation that nuclear YAP localization is induced by osmotic stress. Also, the authors should provide evidence by IF or by nuclear/cytoplasmic fractionation that pYAP-S127 (and pYAP-S128) is present in the nucleus, which would be expected based on the data presented.

5) - The data in Fig 4C showing the effects on YAP-p-S128 by Sorbitol is interesting, but it is unclear why endogenous YAP was not examined. This regulation (ideally with endogenous YAP) should also be shown in another cell type (e.g. MCF10A cells used in Fig EV1A). It would also be prudent to show levels of NLK during this treatment. Is NLK more active or is there more NLK in the cells that cause this response?

6) - A comment about the decreased interaction between 14-3-3 and YAP upon prolonged Sorbitol treatment in Fig 1G should be made. Also, YAP p-S127 levels should be measured in parallel, particularly if they want to conclude "YAP-14-3-3 binding was not increased upon sorbitol treatment despite YAP Ser127 phosphorylation". Similar analysis of YAP p-S127 should also be performed in Fig 2E.

7) - The experiment in Fig 2D should be conducted in parallel with YAP/TAZ knockdown to show that the sorbitol induced expression of CTGF and CYR61 is dependent on YAP/TAZ. This is a critical control for proper conclusions to made from this experiment.

8) - An important control for the kinase assay presented in Fig 3E is the inclusion of GST alone control to determine whether NLK can non-discriminately phosphorylate the GST fusion on YAP. Additionally, a S128A mutant of YAP should be tested in the kinase assay to show that this residue is indeed the target of NLK.

9) - It is curious that there is a shift in the mobility of the YAP S128A mutant in the presence of NLK in Fig 4A. Does this indicate that NLK phosphorylates YAP on residue(s) beyond S128?

10) - Throughout the manuscript the authors state that the kinase assays that are used show direct phosphorylation of the substrate by the immunoprecipitated kinases. Statements regarding the ability for the kinase to directly phosphorylate the substrate cannot be made from immunoprecipitation experiments from mammalian cell lysates as it is possible that another kinase is co-precipitated in the experiments. Accordingly, the word "direct" should be removed from the manuscript text. If the authors want to make conclusions about direct phosphorylation the kinases should be purified using other systems.

11) - It is unfortunate that the authors did not select clones of CRISPR/Cas9-mediated knockout of NLK for their experiments in Figs 3 & 4, as the data would have been much cleaner. Is there a reason why stable clones of NLK knockout cells were used? Was the deletion of NLK toxic to the cells? Given that nuclear YAP can increase cell proliferation the NLK knockout cells should be tested for decreased growth and for decreased expression of YAP target genes.

12) - A control for non-specific binding is missing in Fig 4F. The experiment should be repeated with Flag-YAP and Flag-YAP-S128A expressed without Myc-14-3-3.

13) - The expression of YAP target genes should be tested from the cells used in Fig 5 in the presence and absence of osmotic stress.

14) - Many of the experiments throughout the paper use different cell lines, switching between HEK-293 and other cells with no justification for switching between the different experiments. Also, no information on whether NLK is expressed and to what these levels this might be between the the different cell types.

Referee #3:

1) Hong and colleagues present a manuscript that claims that NLK phosphorylates and activate YAP downstream of osmotic stress. They show that NLK phosphorylates YAP S128 and that this event promotes YAP activity. The analogous residue in Yorkie was shown many years ago by Irvine laboratory to positively regulate Yorkie activity. They showed this using in vivo experiments in flies but unfortunately this important study is not referenced.

2) The current manuscript uses many different cell culture and biochemical studies and for the most part these are thorough and well controlled. The main unresolved issue is: what is the role of NLK and osmotic stress in the regulation of YAP/Hippo in vivo. This is not explored.

3) Furthermore, there is no description of whether S128-YAP phosphorylation normally occurs in cells. Does it happen and the antibody simply can't detect it on endogenous YAP or does it not normally happen?

4) Lastly, the rationale on how they decided to pursue NLK and YAP-S128 are not clear or convincing? This weakens the study.

5) Some aspects of the manuscript need tightening, as indicated below. YAP/TAZ localization under the different treatments should be quantified in Figure 2. The cell images here and elsewhere - eg 4G are often not very clear in terms of the nucleus and cytoplasm. Subcellular fractionation and western blotting would give clearer answers. The images in 4H are clear but many others are not.

6) The figure legends are very wordy and read more like a blend of results/methods.

7) Several parts of the text are not clear. For example: "We found that expression of these two genes was not decreased (actually modestly increased) 4 hours after sorbitol treatment (Fig 1E)." If the statistical analysis found no significant change then is not increased, modestly or otherwise.

8) "YAP nuclear localization was determined in the NLK KO cell pool. YAP nuclear translocation by osmotic stress was blocked in some cells, which presumably had NLK KO, but not other cells, which presumably had the wild-type NLK (Fig 3B)." This is unsatisfactory. Many other explanations are possible. This should be resolved, e.g. with NEK co-staining or by making clones of NEK CRISPR-treated cells that are verified as NEK mutant.

9) They state that when searching for NLK phosphorylation sites in YAP that "We checked YAP amino acids sequence and noticed that YAP Ser 128, which is adjacent to the Ser 127 site, is followed by a proline residue." In fact there are at least 6 "SP" clusters in YAP. What about the other ones? Why was S128 pursued? From the paper, it sounds more like they found out from Moon et al., what the site was and pursued it.

10) "Result showed that NLK expression indeed induced Ser 128 phosphorylation of WT YAP, and this phosphorylation was abolished in YAP S128A mutant (Fig 4A)." This isn't accurate - there is still a band. Is there another YAP-S128 kinase in addition to NLK?

11) 4C and 4D are not very convincing and should be quantified across multiple experiments.

1st Revision - Authors' Response 08 September 2016

Referee #1:

We appreciate the referee for the positive opinion of our study and constructive suggestions.

Hong et al. report in this very interesting manuscript several points that are important for the broader Hippo signalling community. This manuscript is well-presented and the overall implications of the research findings have great potential. However, I think that this manuscript can benefit from some adjustments/extensions as outlined below. I firmly believe that by addressing these points this manuscript will be even more appreciated by the Hippo signalling community and the scientific community at large.

Main points:

1) Correct referencing on page 4. On page 4 the authors state that: "... which is known to promote LATS kinase activity [28]". I am very familiar with the review that is cited as reference 28, so I think that is quite inadequate to be cited in this context. Therefore, I strongly suggest to rather cite instead original publications such as: Praskova M, Xia F, Avruch J. MOBKL1A/MOBKL1B phosphorylation by MST1 and MST2 inhibits cell proliferation. Curr Biol. 2008 Mar 11;18(5):311-21. doi:10.1016/j.cub.2008.02.006. PubMed PMID: 18328708; PubMed Central PMCID: PMC4682548. Hoa L, Kulaberoglu Y, Gundogdu R, Cook D, Mavis M, Gomez M, Gomez V, Hergovich A. The characterisation of LATS2 kinase regulation in Hippo-YAP signalling. Cell Signal. 2016 May;28(5):488-97. doi: 10.1016/j.cellsig.2016.02.012. Epub 2016 Feb 18. PubMed PMID: 26898830.

We thank the reviewer for the insightful suggestion. The original publications are now cited in the revised manuscript.

2) Include statistical analyses to support all "significant" statements. The authors repeatedly state that they observed "significant" changes, which consequently should be backed up by the statistical evaluation of at least three independent experiments. Fortunately, this should be rather easy for Moon et al. to address, since they state in the Methods section (in the subsection "Statistical analysis") that: "Each experiment was repeated three times..." Therefore, please include a statistical analysis of the three independent experiments for:
(i) Figure 1C (change of Lats1 kinase activity)
(ii) Figure 2E (change of 14-3-3/YAP interaction)
(iii) Figure 4D (S128-P in NLK null vs. wild-type cells)
(iv) Figure 4F (change of 14-3-3/YAP interaction) - to match/complement Figure 2E

Quantifications of Western Blot results are included in Figure 1C, 2E, 4D, and 4F. Student's t-tests were used (two-sided, n = 3) to determine the statistical significance.

3) Expand IF studies by biochemical fractionation experiments.
In order to further support their IF studies the authors should include the WB analysis of nuclear/cytoplasmic fractionations for the following figures:
(i) Figure 1D - this is very important and should also include the analysis of S127-P
(ii) Figure 2B - to complement the analysis of Figure 1D
(iii) Figure 3B - to fully establish the importance of NLK

We thank the reviewer for suggesting the fractionation experiments. For point (i), (ii), and (iii), subcellular fractionation experiments were performed and the data are included in Figure EV3D. We are assuming for (ii) the reviewer is asking for Figure 2A, which is YAP localization in no serum condition. If the reviewer is asking for Figure 2B, which is the TAZ localization, we have attached the fractionation data below for reviewer's information. Consistent with the IF data, osmotic stress increased nuclear YAP or TAZ in the absence of serum, and this effect was abolished in NLK KO cells. In contrast, osmotic stress had little effect on the level of nuclear YAP in the presence of serum, again consistent with the IF data. It should be noted that a high level of YAP was found in

the cytoplasmic fraction even in the presence of serum. This is likely due to the leakage of nuclear YAP during fractionation. We also blotted with YAP S127 antibody, however, we didn't see phospho-YAP in the nuclear fraction. This might due to S127-phosphorylated YAP is not present in the nucleus, and/or S128 phosphorylation interferes with pYAP S127 phospho-antibody recognition. We have revised the manuscript accordingly.

(Data not included in the Peer Review Process File)

The YAP/TAZ antibody used in the IF staining is specific as no IF staining is observed in the YAP/TAZ DKO cells (attached below for reviewer's information).

(Data not included in the Peer Review Process File)

(iv) Figure 4H - it would be really important to back up the IF pictures, to fully establish that S128D is a mainly nuclear form irrespective of the conditions.

We performed the fractionation and the result is included in Figure EV4D. Consistent with the IF result, sorbitol did not increase nuclear YAP S128A or S128D mutant. However, likely due to the leakage of nuclear YAP during fractionation, the majority of YAP protein, regardless of WT or mutants, was recovered in the cytoplasmic fraction.

4) Explain the result shown in Figure 4B: According to Figure 4A, the anti-Ser128-P antibody is specific. However, in Figure 4B recombinant YAP is detected by this antibody. How do the authors explain this? Is the antibody not as specific as hoped? Or is Ser128 phosphorylated in bacteria? Or...?

YAP protein should not be phosphorylated in bacterial system because there is no kinase in E. coli. It is likely that the antibody is not as specific as we hoped. This antibody might weakly recognize the unphosphorylated YAP, particularly in the in vitro kinase assay when abundant YAP proteins were used. However, in Figure 4A, we think that the antibody is specific enough for our experiments since NLKinduced YAP phosphorylation in the wild type but not the S128A mutant in vivo. We have revised the text to reflect the above point.

5) Define the Ser127 phosphorylation status of S128A and S128D tested in Figure 4. Considering the very striking IF results shown in Figure 4H, it would be important to also define the Ser127 phosphorylation status of the S128A and S128D mutants of YAP by immunoblotting. Does S127-P negatively correlate with the nuclear localisation of S128D?

We performed YAP S127 phosphorylation Western blot and found that Sorbitol induced YAP S127 phosphorylation in both the YAP S128A and YAP S128D mutant in the presence of serum, which had low basal YAP S127 phosphorylation. YAP localization does not correlate with S127 phosphorylation in the presence of osmotic stress. This is consistent with our model that S128 phosphorylation (mimicked by S128D mutation) is overriding the S127 regulation on YAP localization by interfering 14-3-3 binding. The new data is included at figure EV4E.

6) Re-label and expand Figure 5. Considering that Figure 5 is the main figure regarding the biological relevance of the Ser128 phosphorylation of YAP, I think that this figure needs to be improved.

(i) First of all, all three experiments needed to be presented as n=3 with a corresponding statistical analysis of differences.

We thank the reviewer's suggestion. We have included the statistical analyses in Figure 5B and 5C. In both cases, YAP S128D mutant has lower Sub-G1 phase and apoptotic cells (double positives for 7-AAD and Annexin V) compared with S128A mutant after sorbitol treatment. Student's t-tests were used (two-sided, n = 3).
(ii) The labelling in Figure 5B needs to be adjusted. Instead of "apoptotic" it should state "cell death" or "sub-G1", since this analysis does not allow any conclusion regarding the nature of the cell death as currently is implicated by the labelling.

Labeling has been adjusted accordingly.

(iii) And most importantly (and quite essential), the experiments shown in Figure 5 that are comparing cells expressing YAP WT, S128A or S128D need to be compared to their proper control, which are empty vector expressing cells. - How much proliferation decrease/cell death/apoptosis is altered in cells expressing empty vector alone without any overexpression of any YAP variant?

The comparison between vector and YAP-rescued cells would not be informative because the YAP/TAZ KO cells grow significantly slower than the YAP-rescued cells. The function of YAP/TAZ in cell proliferation/death has been well characterized by many studies. What is relevant to our study is the effect of YAP Ser 128 phosphorylation, therefore, we compared the YAP S128 mutant-rescued cells with the WT-rescued cells.

7) Question regarding the in vitro kinase assay (as described in the Methods section). Why did the authors use mild lysis buffer conditions to measure the activities of Lats1 and NLK? Would it not be better to use stringent buffer conditions to be sure that you are only measuring the kinase at hand and not any potentially co-immunoprecipitating kinase (that it more likely to co-complex in mild conditions than under stringent conditions)?

To address the reviewer's concerns, we have performed LATS1 and NLK in vitro kinase assay using RIPA buffer in cell lysis and immunoprecipitation. In the more stringent RIPA buffer, LATS was activated by sorbitol and only the wild type NLK (NLK-WT) but not the kinase inactive form (NLK-KR) was able to phosphorylate YAP. The data are attached below for reviewer's information.

(Data not included in the Peer Review Process File)

Minor points:

A) Please add page numbers.

B) Add references/text in introduction section. In order to give the non-expert reader a little bit more information in the introduction section, Hong et al. should:

(i) 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.

(ii) Include the two following key publications in the context of the regulation of Lats1/2 by Mst1/2:

Praskova M, Xia F, Avruch J. MOBKL1A/MOBKL1B phosphorylation by MST1 and MST2 inhibits cell proliferation. Curr Biol. 2008 Mar 11;18(5):311-21. doi:10.1016/ j.cub.2008.02.006. PubMed PMID:18328708; PubMed Central PMCID: PMC4682548.

Hoa L, Kulaberoglu Y, Gundogdu R, Cook D, Mavis M, Gomez M, Gomez V, Hergovich A. Thecharacterisation of LATS2 kinase regulation in Hippo-YAP signalling. Cell Signal. 2016 May;28(5):488-97. doi: 10.1016/j.cellsig.2016.02.012. Epub 2016 Feb 18. PubMed PMID: 26898830.

(iii) Describe briefly the S127 and S381 (S397) phosphorylation of YAP as regulatory mechanisms (including some key references).

Revisions have been made to address each of the above points.

Referee #2:

We appreciate the referee for the positive opinion of our study and constructive suggestions.

The manuscript by Hong et al outlines a mechanism by which osmotic stress, which can be induced by the introduction of high levels of Sorbitol or NaCl in cell growth media, induces the nuclear localization and activity of the Hippo pathway effectors YAP and TAZ. The authors describe a mechanism by which phosphorylation of YAP on Ser128 upon osmotic stress increases nuclear YAP localization and activity. Ser128 lies adjacent to the well characterized Ser127 within YAP, which is phosphorylated in response to Hippo pathway activity and controls YAP binding to 14-3-3, which is a major mechanism thought to induce YAP cytoplasmic restriction. The authors propose that phosphorylation of S128 disrupts 14-3-3 binding and thereby increase nuclear YAP. The authors also suggest that NLK is the kinase that mediates YAP-S128 phosphorylation in response to osmotic stress, and provide evidence that mutation of Ser128 within YAP leads to increased nuclear YAP activity. The manuscript outlines a new biological mechanism for YAP/TAZ regulation and identifies a novel kinase that controls YAP activity, which together make this manuscript conceptually interesting. However, several things need to be addressed before the data is suitable for publication, most of which are outlined below:

1 - The proposed mechanism of osmotic stress regulating YAP/TAZ phosphorylation/activity is inconsistent with the data published by the same group. A previous publication from the Guan Lab (Mo et al, 2015) Supplemental Figure S1 shows 25mM and 50mM Sorbitol shows no changes in YAP phosphorylation, which contradicts the data presented in Fig1A. What is the explanation for this? Can the authors demonstrate at what concentration (below 0.2M used in this submission) of Sorbitol alters the phosphorylation of Yap? Why would altered levels of osmotic stress impact YAP differentially?

The current data are consistent with previous observations. In Fig1A, cells were treated with 0.2 M Sorbitol. We have tested various concentrations of sorbitol and found that sorbitol lower than 50 mM had no effect on YAP phosphorylation and 100 mM sorbitol had a minor effect. Only when sorbitol concentration is at 200 mM or higher, it induced YAP phosphorylation as well as LATS phosphorylation. The new data are included as figure EV1A.

2) - Osmotic stress has long been known to impact actin cytoskeleton remodeling, and therefore given the relationship between actin regulation and YAP/TAZ localization it is possible that changes in the actin cytoskeleton mediate the observed changes described by the authors. The authors should determine how the concentrations of Sorbitol and NaCl impact the actin cytoskeleton in the cells that they are using, and determine whether these potential changes impact YAP/TAZ. It would also be very interesting to examine whether NLK-mediated phosphorylation of YAP/TAZ is regulated by actin changes.

We thank the reviewer for the comments, but feel that this may be beyond the scope of the current study. We have revised the manuscript to discuss this point in the discussion section.

3) - Quantitation of all the presented immunofluorescence experiments need to be included.

IF quantifications are added in the figures.

4) - The authors should show by biochemical fractionation that nuclear YAP localization is induced by osmotic stress. Also, the authors should provide evidence by IF or by nuclear/cytoplasmic fractionation that pYAP-S127 (and pYAP-S128) is present in the nucleus, which would be expected based on the data presented.

We thank the reviewer for suggesting the fractionation experiments. Subcellular fractionation experiments were performed and the data are included in Figure EV3D. Consistent with the IF data, osmotic stress increased nuclear YAP in the absence of serum, and this effect was abolished in NLK KO cells. In contrast, osmotic stress had little effect on the level of nuclear YAP in the presence of serum, again consistent with the IF data. It should be noted that high level of YAP was found in the

cytoplasmic fraction even in the presence of serum. This is likely due to the leakage of nuclear YAP during fractionation. We also blotted with YAP S127 antibody and did not see phospho-YAP in the nuclear fraction. This might be due to S127-phosphorylated YAP is not present in the nucleus, and/or S128 phosphorylation is interfering with pYAP S127 phospho-antibody recognition. The YAP S128 phosphoantibody is rather weak and we were unable to detect YAP S128 phosphorylation signal in the nuclear fraction. We have revised the manuscript accordingly.

5) - The data in Fig 4C showing the effects on YAP-p-S128 by Sorbitol is interesting, but it is unclear why endogenous YAP was not examined. This regulation (ideally with endogenous YAP) should also be shown in another cell type (e.g. MCF10A cells used in Fig EV1A). It would also be prudent to show levels of NLK during this treatment. Is NLK more active or is there more NLK in the cells that cause this response?

Actually, S128 phosphorylation of immunoprecipitated endogenous YAP was presented in the original figure. We are sorry that the figure was not clearly labeled. We have rearranged the figure to make it clear regarding the data from endogenous YAP and transfected Flag-YAP (Fig.4C). According to the reviewer's suggestion, we have tested the phosphorylation of YAP S128 in MCF10A cells. Similar to HEK293A cells, osmotic stress increased phosphorylation of endogenous YAP S128 in MCF10A cells (Figure EV4B). NLK level is unchanged upon osmotic stress treatment (shown below), but its activity has been shown to be induced by osmotic stress in our previous study [1].

(Data not included in the Peer Review Process File)

6) - A comment about the decreased interaction between 14-3-3 and YAP upon prolonged Sorbitol treatment in Fig 1G should be made. Also, YAP p-S127 levels should be measured in parallel, particularly if they want to conclude "YAP-14-3-3 binding was not increased upon sorbitol treatment despite YAP Ser127 phosphorylation". Similar analysis of YAP p-S127 should also be performed in Fig 2E.

We have commented the decreased YAP and 14-3-3 interaction in the revised manuscript. We examined YAP Ser 127 phosphorylation status under these experimental conditions. As expected, YAP S127 phosphorylation was increased. The data is included for reviewer's information, but not added to the manuscript because the same point of increased YAP S127 phosphorylation by osmotic stress is shown in Fig 1A.

(Data not included in the Peer Review Process File)

7) - The experiment in Fig 2D should be conducted in parallel with YAP/TAZ knockdown to show that the sorbitol induced expression of CTGF and CYR61 is dependent on YAP/TAZ. This is a critical control for proper conclusions to made from this experiment.

We have performed the experiments with the YAP/TAZ dKO cells as controls. Our result shows that CTGF and Cyr61 expression can only be induced in WT cells but not the YAP/TAZ dKO cells (two-sided student's t-test, n = 3) (Figure 2D), suggesting that the induction of CTGF and Cyr61 by sorbitol is YAP/TAZ dependent.

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8) - An important control for the kinase assay presented in Fig 3E is the inclusion of GST alone control to determine whether NLK can non-discriminately phosphorylate the GST fusion on YAP. Additionally, a S128A mutant of YAP should be tested in the kinase assay to show that this residue is indeed the target of NLK.

According to the reviewer's suggestion, we have included GST alone as a control to make sure NLK phosphorylates YAP protein but not GST. Our result (see attached below) shows that GST was not phosphorylated by NLK (left panels). We have also tried in vitro kinase assay using GST-YAP S128A mutation. GST-YAP S128A mutant could still be phosphorylated by NLK (see attached below, right panels), suggesting that NLK can phosphorylate YAP on additional sites. This result is expected because YAP has 10 putative NLK phosphorylation consensus sites SP or TP. The data in Figure 4A and 4B have shown that YAP Ser 128 is an NLK target site both in vitro and in vivo.

(Data not included in the Peer Review Process File)

9) - It is curious that there is a shift in the mobility of the YAP S128A mutant in the presence of NLK in Fig 4A. Does this indicate that NLK phosphorylates YAP on residue(s) beyond S128?

Consistent with the reviewer's suggestion that besides S128, NLK can phosphorylate YAP on additional residues (see response to question 8). We noted this point in the revised manuscript.

10) - Throughout the manuscript the authors state that the kinase assays that are used show direct phosphorylation of the substrate by the immunoprecipitated kinases. Statements regarding the ability of the kinase to directly phosphorylate the substrate cannot be made from immunoprecipitation experiments from mammalian cell lysates as it is possible that another kinase is co-precipitated in the experiments. Accordingly, the word "direct" should be removed from the manuscript text. If the authors want to make conclusions about direct phosphorylation the kinases should be purified using other systems.

We thank the reviewer for pointing out the issue. To address this, we have repeated the experiments using a more stringent RIPA buffer in the immunoprecipitation and included kinase dead mutant as a control. Our results show that immunoprecipitated LATS and NLK can phosphorylate YAP (see data attached below). These data support a notion that LATS and NLK directly phosphorylate YAP. However, we could not unequivocally exclude the possibility that a kinase that was co-precipitated with wild type NLK but not the kinase dead mutant was responsible for YAP phosphorylation. We have removed the word "direct."

(Data not included in the Peer Review Process File)

11) - It is unfortunate that the authors did not select clones of CRISPR/Cas9-mediated knockout of NLK for their experiments in Figs 3 & 4, as the data would have been much cleaner. Is there a reason why stable clones of NLK knockout cells were used? Was the deletion of NLK toxic to the cells? Given that nuclear YAP can increase cell proliferation the NLK knockout cells should be tested for decreased growth and for decreased expression of YAP target genes.

We had generated NLK KO clones, but found that the properties of stable NLK KO clones changed during passage. It is possible that NLK KO cells are under constant stress and the cells may slowly develop adaptive responses. That is why we chose to use transient knockout cells. Another advantage for the transient knockout experiment is that the wild type cells and KO cells were cultured under identical conditions.

12) - A control for non-specific binding is missing in Fig 4F. The experiment should be repeated with Flag- YAP and Flag-YAP-S128A expressed without Myc-14-3-3.

We apologize for the insufficient control in this experiment. We have presented data including the control of without Myc-14-3-3 (Figure 4F).

13) - The expression of YAP target genes should be tested from the cells used in Fig 5 in the presence and absence of osmotic stress.

We thank for the reviewer's suggestion. But we feel this information is not necessary for the conclusion of the figure that S128 phosphorylation affects YAP localization.

14) - Many of the experiments throughout the paper use different cell lines, switching between HEK-293 and other cells with no justification for switching between the different experiments. Also, no information on whether NLK is expressed and to what these levels this might be between the different cell types.

We have mostly used HEK293A cells in our study (virtually for all experiments). We also used MCF10A to support the idea that this regulation is not restricted to HEK293A cells only. NLK levels in HEK293A and MCF10A cells are similar (see the attachment in response to comment #5). Based on the protein atlas database, NLK is widely expressed in many cell types.

Referee #3:

We appreciate the referee for the positive opinion of our study and constructive suggestions.

1) Hong and colleagues present a manuscript that claims that NLK phosphorylates and activate YAP downstream of osmotic stress. They show that NLK phosphorylates YAP S128 and that this event promotes YAP activity. The analogous residue in Yorkie was shown many years ago by Irvine laboratory to positively regulate Yorkie activity. They showed this using in vivo experiments in flies but unfortunately this important study is not referenced.

We thank the reviewer for the insightful comment. We have discussed the drosophila study and cited the reference, which is consistent with our study, in the revised discussion section.

2) The current manuscript uses many different cell culture and biochemical studies and for the most part these are thorough and well controlled. The main unresolved issue is: what is the role of NLK and osmotic stress in the regulation of YAP/Hippo in vivo. This is not explored.

We have examined the osmotic-dependent YAP regulation in cultured cells. However, we feel that in vivo mouse experiment is beyond the scope of the current study. Luckily, as pointed out by the reviewer's comment #1, previous study from Ken Irvine's group provides supporting in vivo functional data from drosophila.

3) Furthermore, there is no description of whether S128-YAP phosphorylation normally occurs in cells.
Does it happen and the antibody simply can't detect it on endogenous YAP or does it not normally happen?

We showed in Figure 4C that osmotic stress increased S128 phosphorylation of endogenous YAP. We apologize that the original figure 4C was not clearly labeled. We have rearranged the figure to make the endogenous YAP S128 phosphorylation data obvious. In addition, according to the online database phosphosite.org website, YAP S128 phosphorylation has been detected by many phosphoproteomic studies. Thus, YAP S128 is likely to be phosphorylated in vivo.

4) Lastly, the rationale on how they decided to pursue NLK and YAP-S128 are not clear or convincing. This weakens the study.

We apologize for not making it clear in the original manuscript. We pursued NLK because inhibition of p38 and JNK, two known osmotic stress-activated MAP kinases, had no effect. Furthermore, we know that NLK is activated by osmotic stress [1]. Therefore we tested NLK. NLK is a proline directed kinase; we paid attention to NLK phosphorylation consensus sites in YAP. We also noticed that YAP is in the nucleus even though it is highly phosphorylated. It is well known that YAP S127 phosphorylation is responsible for YAP nuclear-cytoplasmic shuttling. We were intrigued by the fact that S128 is a NLK consensus site and in the 14-3-3 binding region. In addition, we learned at the 2015 Keystone Hippo symposium that Dr. Jho had developed phospho-YAP S128 antibody. We have revised the manuscript to make these rationales behind our experiments more obvious to the readers.

5) Some aspects of the manuscript need tightening, as indicated below.
YAP/TAZ localization under the different treatments should be quantified in Figure 2. The cell images here and elsewhere - eg 4G are often not very clear in terms of the nucleus and cytoplasm. Subcellular fractionation and western blotting would give clearer answers. The images in 4H are clear but many others are not.

Quantification for IF result is included in the revised figures. As for the quality of figures, we have repeated the experiment and included the new data for figure 2C. Subcellular fractionation is added in the revised figures (Fig EV3D).

6) The figure legends are very wordy and read more like a blend of results/methods.

We apologize for the poor wording in the figure legends. We have revised the figure legends to make

them more concise.

7) Several parts of the text are not clear. For example: "We found that expression of these two genes was not decreased (actually modestly increased) 4 hours after sorbitol treatment (Fig 1E)." If the statistical analysis found no significant change then is not increased, modestly or otherwise.

The change in CTGF and CYR61 expression level in the presence of serum is not significant (p-value >0.05). However, in each of these three experiments, we observed a small increase in CTGF and CYR61 expression levels. As a result, we cannot conclude that there is a change in target gene expression by osmotic stress in this condition, due to the lack of statistical support. We have revised the text.

8) "YAP nuclear localization was determined in the NLK KO cell pool. YAP nuclear translocation by osmotic stress was blocked in some cells, which presumably had NLK KO, but not other cells, which presumably had the wild-type NLK (Fig 3B)." This is unsatisfactory. Many other explanations are possible. This should be resolved, e.g. with NEK costaining or by making clones of NEK CRISPR-treated cells that are verified as NEK mutant.

We found that the properties of stable NLK KO clones changed during passage, therefore it is difficult to analyze the stable KO clones. It is possible that NLK KO cells are under constant stress and the cells may slowly develop adaptive responses. That is why we choose to use transient knockout cells. Another advantage for the transient knockout experiment is that the wild type cells and KO cells were cultured under identical conditions.

We have tried, but unable to find an NLK antibody suitable for immunoflourescence staining. According to the Western blot result (Fig.EV3B), an efficient NLK knockout was achieved by the transient transfection of CRISPR/CAS9. Quantification of the IF data shows that YAP nuclear localization is dramatically decreased in the majority of cells of the NLK KO pool when compared to the control cells (Fig. 3B). Taken together, our data support a notion that NLK is involved YAP nuclear-cytoplasmic shuttling in response to osmotic stress.

9) They state that when searching for NLK phosphorylation sites in YAP that "We checked YAP amino acids sequence and noticed that YAP Ser 128, which is adjacent to the Ser 127 site, is followed by a proline residue." In fact there are at least 6 "SP" clusters in YAP. What about the other ones? Why was S128 pursued? From the paper, it sounds more like they found out from Moon et al., what the site was and pursued it.

S128 is pursued because it is close to the YAP-14-3-3 binding pocket, therefore we suspect that the phosphorylation on this site might contribute to the reduced 14-3-3 binding and thus YAP localization. In addition, we learned at the Keystone Hippo symposium that Dr. Jho has developed phospho-YAP S128 antibody. As a collaboration, we tested whether YAP S128 phosphorylation was increased upon osmotic stress. We have revised the manuscript to make these logics/rationales more clear to readers.

10) "Result showed that NLK expression indeed induced Ser 128 phosphorylation of WT YAP, and this phosphorylation was abolished in YAP S128A mutant (Fig 4A)." This isn't accurate - there is still a band. Is there another YAP-S128 kinase in addition to NLK?

The weak signal detected by the YAP S128 phosphoantibody could be due to the action of endogenous
NLK or a weak recognition of unphosphorylated YAP by the antibody. We cannot exclude the possibility that there are additional YAP S128 kinases in the cells. We have revised the manuscript to note the above point.

11) 4C and 4D are not very convincing and should be quantified across multiple experiments.

Quantifications are included in the figures (Figure 4C and 4D). Student's t-test was performed and a significant increase of YAP Ser 128 phosphorylation was observed in HEK293A cells in response to sorbitol treatment (Figure 4C). This induction of YAP Ser 128 phosphorylation was deminished in NLK KO cells (Figure 4D).

Reference: 1. Yuan HX, Wang Z, Yu FX, Li F, Russell RC, Jewell JL, Guan KL (2015) NLK phosphorylates Raptor to mediate stress-induced mTORC1 inhibition. *Genes & development* **29**: 2362-76

2nd Editorial Decision 06 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, before we can proceed with formal acceptance, I have a few editorial requests.

Your manuscript has currently 5 figures and 4 EV figures. However, figures 4 and 5 have are currently multi-page format, which does not fit to the requirements of our publisher. Please prepare the figure files according to these guidelines and upload these as single high-resolution files in TIFF or EPS format.

For a scientific report we only allow 5 figures and the results and discussion sections must be combined. If you whish to publish this as scientific report, then you need to fit your data into 5 single page figures and not more than 5 EV figures. Additional data could be shown in an Appendix (see below). Otherwise, I suggest publishing this as an article.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Please update all the call-outs in the manuscript when you change the figures!

Please also provide a legend for Figure EV4E.

The scale bars in the microscopic images differ in thickness. Please provide clear to see scale bars of similar thickness for these panels.

REFEREE REPORTS

Referee #1:

The authors have sufficiently addressed all my concerns by significantly expanding the originally manuscript by responding to nearly all of my points experimentally.

Referee #2:

This revised manuscript by Hong et al. is much improved and the authors have addressed most of my concerns. The manuscript is suitable for publication.

Referee #3:

The in vivo significance of YAP-S128 phosphorylation by NLK has not been tested and is still

unknown. The authors have responded to almost all other reviewer suggestions and as such the manuscript is significantly improved.

2nd Revision - Authors' Response 07 November 2016

The authors made the requested changes and submitted updated versions of the manuscript and applicable files.

3rd Editorial Decision – Acceptance 11 November 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.

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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?
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  - definition of 'center values' as median or average;
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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?	No pre-determination of sample sizes was done. Instead, biological replicates for experiments were done for at least three times to support the statement of significant differences.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We do not have animal studies.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We do not have animal studies.
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4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Not applicable. Because no animal studies have been included, no doube-blind or similar steps have been done.
4.b. For animal studies, include a statement about blinding even if no blinding was done	We do not have animal studies
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. We used Student's t-test with two sample unequal variance (non-normal distribution).
Is there an estimate of variation within each group of data?	No
Is the variance similar between the groups that are being statistically compared?	No

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).	A list of antibodies can be found in the Material s and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The cell lines are from ATCC. Thye have not been authenticated. However, they have been tested for mycoplasma and free of this infection.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	We do not have animal studies.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	We do not have animal studies.
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.	We do not have animal studies.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	We do not have human subjects.
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14. Report any restrictions on the availability (and/or on the use) of human data or samples.	We do not have human subjects.
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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.
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G- Dual use research of concern

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