EMBO reports

TRIP6 inhibits Hippo signaling in response to tension at adherens junctions

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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 17 August 2017

Thank you for the submission of your research manuscript to EMBO reports. I apologize for the rather slow review process, but due to the summer holidays season it took longer than normal to find referees for the manuscript, and also to get the reports back from the reviewers. But, we have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees support the publication of your paper in EMBO reports. Nevertheless, they all have a number of concerns and/or suggestions to improve the manuscript, which we ask you to address in a revised manuscript. As the reports are below, I will not detail them here, also as I think that all points should be addressed.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in a 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

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.

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

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

REFEREE REPORTS

Referee #1:

Mechanical cues can induce a fast translocation of specific transcriptional co-factors such as YAP and TAZ from the cytoplasm to the cell nucleus where they activate specific transcriptional programs. Under high mechanical forces, YAP localize to the nucleus while low tension causes YAP retention in the cytosol. The Hippo signaling pathway regulates cellular signaling in response to mechanical input in part through LATS1/2-mediated phosphorylation and inhibition of YAP. However, there is still a considerable lack of knowledge how mechanical cues such as tension are sensed and transduced. Here, Dutta and colleagues show that TRIP6, a LIM domain containing protein of the zyxin family, enhances YAP nuclear localization and activity by inhibiting LATS1/2 kinases. Interaction of TRIP6 with LATS1/2 competes with MOB1/LATS interaction and thereby inhibits the recruitment of MST1/2. Importantly, the authors also show that TRPI6 is recruited to adherens junctions in a vinculin- and tension-dependent manner. Vinculin binds TRIP6 and stimulates its binding to LATS1/2.
The manuscript reports interesting and important findings that add to our understanding of endothelial cell function. The paper is clearly written and the study is very systematic and thorough. However, there are several issues that should be addressed to strengthen the manuscript. These include the mechanism of how vinculin regulates TRIP6-LATS1/2 interaction and signaling as well as the question if this pathway is also functioning in mesenchymal cells.

1) Figure 1C shows the interaction of TRIP6 with two segments within the N-terminal region of LATS2. It is not entirely clear why the authors analyzed these two specific regions. Probably because of the similar interaction motives of Ajuba and Zyxin with LATS but this could be better stated in the text. Have the authors determined if the amino acid segments 376-397 and 625-644 are the only interaction interfaces between TRIP6 and LATS2 by expressing LATS2 deletions lacking these regions? Such deletion variant could also serve as control for Figure 2A to show that the reduced MOB1-LATS2 interaction in the presence of TRIP6 is due to a direct TRIP6-LATS2 interaction.
2) Some of the localization studies (Figure 3, Figure 5) would benefit from showing co-stainings with E-cadherin to show the integrity of the cell-cell contacts. The authors write that "TRIP6 and LATS1/2 affect each other's localization" - while this is convincingly shown that LATS does not properly localized to adherens junctions in the absence of TRIP6, it is less obvious if TRIP6 localization depends on LATS1/2. In Figure 3B, the punctuate staining could indeed reflect changes in cell-cell adhesion.
3) The authors suggest that vinculin promotes TRIP6-LATS interaction either directly or indirectly by inducing a conformational change in TRIP6. Figure 2B shows that TRIP6 binds LATS2 in the absence of vinculin. To test the hypothesis they could use the experimental setup of Figure 2B and add recombinant vinculin (either wildtype or an activated form bearing mutations that inhibit head-tail association (Cohen et al., 2005)) to show if vinculin has a direct effect on TRIP6-LATS interaction. Expression of an activated vinculin form in MCF10A cells would also allow the authors

to address the question of tension promotes vinculin-TRIP6 interaction by activating vinculin.
4) Figure 5E: Vinculin depletion reduces LATS and TRIP6 localization to adherens junctions and YAP localization and activity. Is this a direct effect due to interfering of the vinculin-TRIP6-LATS pathway or an indirect effect as vinculin-depleted cells might exhibit less cellular tension? Expression of a vinculin binding-deficient TRIP6 variant would help to address this point. Alternatively, have the authors looked at TRIP6 localization in vinculin-depleted cells after cells stretch?
5) Two questions additional general questions arise from this study. Addressing these two questions might be out of the scope of the revisions but would strengthen the manuscript.
Since vinculin and TRIP6 also localize to cell-matrix adhesions (focal adhesion) the work by Dutta et al. raises the question if the vinculin-TRIP6-LATS1/2 axis is also active in mesenchymal cells to regulate cellular responses to mechanical cues.
Another intriguing is if zyxin and ajuba, which bind to a similar region within LATS, might also function during mechanotransduction by blocking MOB1/LATS interaction and thereby inhibiting the recruitment of MST1/2. Do the authors have any indication if this mechanism is conserved among zyxin protein family members?

Minor points:
1) Figure 1H: It is not clear why MST2 was overexpressed in this experiment. Smaller but still significant differences in MST2 T180 phosphorylation might be masked by overexpression of MST2.
2) Figure 2A, blot description is unclear. In the current form, they have two blots for GFP-LATS2 after IP. I assume the middle blot shows the GFP-LATS2 levels in the lysate and not after myc-IP. Figure 2A, quantification: GFP-LATS2 is mention twice in the x-axis description, while MOB1-Myc is missing.

Referee #2:

This study addresses the important question of how tension regulates growth and proliferation factor signaling. The authors present evidence that Trip6 inhibits Lats1/2 at adherens junctions, thereby relieving inhibition of YAP by Lats1/2. They show that Trip6 competes with Mob1 for a binding site on Lats1/2. They extend their analysis to examine what role an interaction between Trip6 and vinculin might play in the mechanoresponsive regulation of the Trip6-Lats1/2 interaction. The study is well executed, and clearly presented. The data regarding the Trip6-Lats1/2 interaction is thorough and convincing, as is the data showing Trip6 competes with Mob1. On the other hand, the data suggesting an interaction between Trip6 and vinculin is less convincing.
Specific concerns-
It would be helpful to readers who are not YAP/TAZ aficionados if you included a model diagram showing how the proteins of interest in the pathway interact, and how the new data presented fits in.
In the last sentence of the first paragraph in the Results section, you say your Co-IP results are "highly reminiscent" of those for other zyxin family members. Please be specific about which results. Additionally, zyxin has been shown to interact with and regulate Lats2. Presentation of this in the Introduction (you do mention one paper in the Results) would be helpful in presenting a more general role for Lim domain proteins in the regulation of Hippo signaling.
This also raises the concern that by overexpression of Trip6, you may be competing with an interaction with with Lats1/2 and another protein such as zyxin or ajuba. What proteins are in the immune complex when you pull down Lats2? It would be informative to show something like a silver stain of the recovered complex, not just antibody labeling of the studied proteins, so we have a sense of how stringent the conditions are, and how specific the interaction is.
All of the straining where you look at localization, or lack thereof, to cell-cell junctions or to focal adhesions should have a counter stain to show the structures are intact and that you are in the correct focal plane. (3F, 4A, 5B, C, E and G) You effectively utilize cadherin and FAK for this in some panels, but your image data would be more convincing if you used it in all panels. This would provide an opportunity to extend your quantitation of the localization through colocalization analysis. To be specific, is the change in Trip6 distribution in Figure 3B the result of an incomplete distribution within the cell-cell junctions, or has the KD of Lats1 and 2 actually altered the morphology of the junctions? This is important to demonstrate your assertion that 'Trip6 and Lats1/2 affect each other's localization.' That said, I appreciate the quantitation you did do, for example, in Figures S3D and E.

You assert Trip6 is part of a mechanosensory complex. Mechanosensation is when a protein actually fells the stress and experiences strain that causes bonds to break, helices to unravel, etc. What you observe is changes in localization and activation as a result of force input. This is mechanoresponse. Think smoke detector for sensation, fire department for response. I am aware that this is not a uniform standard in our area of interest, but it is an important distinction.
Your assertions about what your data demonstrates in regards to the vinculin-Trip6 interaction and regulation thereof are overstated. While I agree that an inference can be made, you go beyond this in the final sentences of the Results section. High throughput Y2H and presence in the immune complex are not convincing evidence of a primary interaction. Again, we have no idea what else is in the immune complex since you have only presented antibody labeling of the proteins of interest. A silver stained gel would be informative, and a rigorous mutational analysis, like you performed with Trip6 and Lats2 would be required for this. Additionally, since you do not provide any staining showing intact adhesion structures in Figure 5G and E, I wonder if the adhesions have been disrupted and the changes you are seeing in protein levels, localization and downstream changes in YAP activity are secondary to this. I think that the role of vinculin on regulation of Trip6 and Lats is an important finding, if true, and deserves a shoring up of the data.

Referee #3:

The manuscript by Dutta et al, identifies TRIP6 as a novel upstream regulator of the Hippo kinase cascade that inhibits LATS kinase activity by competing with MOB for LATS binding. The authors demonstrate that TRIP6 is localized at cell-cell junctions and modulate LATS kinases in response to dynamic changes in physical tension at cell junctions. This is supported by showing dynamic changes in co-localization and physical interaction between LATS and TRIP6 in response to various upstream stimuli such as changes in cell density or actin cytoskeleton remodelling. Furthermore Vinculin has been identified to relay mechano-sensing stimuli to the LATS kinases by interacting with and regulating TRIP6 localization. Altogether the data presented in this manuscript connects Vinculin-TRIP6 to LATS1/2 kinases and provides a novel mechanism for regulation of the Hippo kinase cascade downstream of cell-cell contact and mechanotransduction. However, the authors should provide further evidence for some of the suggested mechanisms to make a stronger case for the proposed model.

The authors propose that TRIP6 inhibits LATS1/2 kinases by competing with MOB for LATS binding. This is supported by a CoIP experiment in which the expression of TRIP6 has been shown to reduce LATS binding (Fig 2A) and an in vitro competition binding assay (Fig 2B). To provide more evidence for this mechanism, the authors could also investigate the effect of TRIP6 mutant construct that is deficient in LATS binding (1-277), in the LATS-MOB CoIP experiment, which presumably would not decrease LATS-MOB binding upon expression.

In Figure 5, Vinculin has been shown to interact with and recruit TRIP6 to cell-cell junctions and this is proposed as a mechanism through which tension at cell junctions regulate LATS1/2 kinases. Although TRIP6 interaction with LATS and Vinculin has been investigated separately, it is important to demonstrate concomitant binding and complex formation between TRIP6, LATS and Vinculin in a CoIP experiment to further support this model. It would also make a stronger case for the proposed mechanism to demonstrate a decrease in protein interaction between TRIP6-LATS-Vinculin in response to at least one of the conditions that reduces TRIP6-Vinculin and TRIP6-LATS binding (i.e. high cell density or loss of actin stress fiber). The authors might also look at LATS phosphorylation (S909/T1079) upon Vinculin loss of function.

Since the authors propose a LATS-dependent model for TRIP6 mechanism of action it would be nice, though not critical, to investigate the effect of TRIP6 depletion on subcellular localization of YAP mutants that are refractory to LATS inhibition (YAP-S127 or YAP5SA).

Minor errors/comments:

In Fig. 1b, the labels are mis-positioned.

In Fig S1: Which shRNA was used in panel a?

There is a labelling error in Figure 2A in distinguishing the IP samples and total lysate. GFP-LATS2 on top of the lysate samples has mistakenly been identified as IP sample.
To refer to TRIP6 loss of function (either by CRISPR or RNAi), the authors use TRIP6Δ in the manuscript and in the figures. This is not common and may cause confusion by implying that TRIP6Δ is a TRIP6 mutant construct (i.e. a dominant negative version). Depending on the experiment and whether shRNA or CRISPR-Cas system has been used for loss of function, the authors could use the pertinent and clearer terminology such as TRIP6-KD (Knockdown) or TRIP6-KO (Knockout).

1st Revision - authors' response 19 October 2017

Point by point response to reviewer comments

Reviewer #1

1) As suggested by the reviewer, we now clearly state in the text that the two regions of LATS2 (amino acids 376-397 and 625-644) that we tested for TRIP6 binding were based on the regions of LATS1/2 previously defined as responsible for interacting with the TRIP6 family members Ajuba and Zyxin. The reviewer also as suggested deleting these regions of LATS2 and testing if TRIP6 was now unable to compete with MOB1 for binding to LATS2. Because the second domain of LATS2 (625-644) is in the middle of the MOB1 binding site and would disrupt MOB1-LATS2 binding, we tested whether deletion of amino acids 376-397 of LATS2 disrupted TRIP6 binding to LATS2. We found that deletion of these amino acids in the context of the full length LATS2 did not disrupt TRIP6-LATS2 binding. Thus, we were unable to do the competition experiment using this version of LATS2. However, we did a related experiment suggested by Reviewer #3 and showed (Figure 2A) that unlike full length TRIP6, a TRIP6 mutant (1-277) that cannot bind LATS2 was unable to compete with MOB1 for binding to LATS2.

2) The reviewer is interested in whether knockdown of TRIP6, LATS1/2, or Vinculin affects cell junctions as judged by E-Cadherin staining. We have done this staining (Figure EV4C) and while we cannot rule out that there are subtle changes in junction architecture (we state this in the text), we do not see any obvious change in E-Cadherin staining. In addition, we tested whether knockdown of TRIP6 in cells grown to high density interferes with recruitment of Vinculin to cell-cell junctions following stretch. We observe that Vinculin is recruited normally to cell-cell junctions in a tension dependent manner in TRIP6 depleted cells. This result is consistent with our other data suggesting that TRIP6 acts downstream of Vinculin and that TRIP6 depletion is not radically disrupting adherens junctions in MCF10A cells.

3) The reviewer states: "*The authors suggest that vinculin promotes TRIP6-LATS interaction either directly or indirectly by inducing a conformational change in TRIP6. Figure 2B shows that TRIP6 binds LATS2 in the absence of vinculin. To test the hypothesis they could use the experimental setup of Figure 2B and add recombinant vinculin (either wildtype or an activated form bearing mutations that inhibit head-tail association (Cohen et al., 2005)) to show if vinculin has a direct effect on TRIP6-LATS interaction. Expression of an activated vinculin form in MCF10A cells would also allow the authors to address the question of tension promotes vinculin-TRIP6 interaction by activating vinculin.*"

Response: We agree that it would be nice to be able to reconstitute the vinculin stimulated TRIP6-LATS2 binding reaction in vitro. Indeed this is the direction we are moving towards, but feel that this work is beyond the scope of the present study, at least in part because there may be additional players needed to reconstitute the system (see below). However, we have followed the reviewer's alternative suggestion by testing whether the an activated "open" conformation mutant in vinculin (vinculin-T12) is better at binding TRIP6 than wild-type vinculin. We did this experiment by overexpressing TRIP6, LATS2, and either wild-type or T12 vinculin in HEK293 cells and then immunoprecipitating TRIP6 and blotting for vinculin and LATS2. These experiments (Figure 6G) showed that more vinculin-T12 than wild-type vinculin came down with TRIP6, consistent with the T12 open form of vinculin being better at forming a complex with TRIP6. Surprisingly, we did not see a change in the amount LATS2 coming down with TRIP6. Several possible explanations for these results come to mind. One possibility is that vinculin-T12 does not perfectly mimic the open

form of vinculin at adherens junctions under tension and although it binds better to TRIP6, it is unable to stimulate TRIP6 binding to LATS1/2. Alternatively, one or more other proteins besides vinculin might be needed to enhance TRIP6 binding to LATS2 in response to tension. So although vinculin is required to stimulate TRIP6-LATS1/2 binding in vivo, it might not sufficient. These possibilities are now mentioned in the Results and Discussion.

4) The reviewer states. "*Figure 5E: Vinculin depletion reduces LATS and TRIP6 localization to adherens junctions and YAP localization and activity. Is this a direct effect due to interfering of the vinculin-TRIP6-LATS pathway or an indirect effect as vinculin-depleted cells might exhibit less cellular tension? Expression of a vinculin binding-deficient TRIP6 variant would help to address this point. Alternatively, have the authors looked at TRIP6 localization in vinculin-depleted cells after cells stretch?*"

Response: The reviewer raises a good point. We have done the alternate experiment suggested by the reviewer and discovered that TRIP6 is unable to localize to cell-cell junctions in vinculin depleted cells after stretch consistent with our model (Figure 6H).

5) Reviewer comment: "*Two questions additional general questions arise from this study. Addressing these two questions might be out of the scope of the revisions but would strengthen the manuscript. Since vinculin and TRIP6 also localize to cell-matrix adhesions (focal adhesion) the work by Dutta et al. raises the question if the vinculin-TRIP6-LATS1/2 axis is also active in mesenchymal cells to regulate cellular responses to mechanical cues.*
Another intriguing is if zyxin and ajuba, which bind to a similar region within LATS, might also function during mechanotransduction by blocking MOB1/LATS interaction and thereby inhibiting the recruitment of MST1/2. Do the authors have any indication if this mechanism is conserved among zyxin protein family members?"

Response: We agree that both of those questions are quite interesting but we feel that they are beyond the scope of the present work. That said, we have looked at fibroblasts and do not see any clear localization of LATS1/2 to cell junctions or focal adhesions, and we never observe LATS1 at focal adhesions in epithelial cells. As for zyxin and ajuba, previous studies indicate that they bind to similar regions of LATS1/2 so it is entirely plausible that they could act in the same way, but we have not tested this directly. We are aware, (through personal communication with the research group involved) of a story under review indicating that an ajuba family member is involved in tension dependent regulation of Hippo signaling. So there will likely be more coming out on other family members.

Minor Points
1) The reviewer asks why MST2 is overexpressed in figure 1H where we test whether TRIP6 affects MST1 phosphorylation at T180. We have tried the experiment looking at the endogenous protein but we are unable to detect phosphorylation on T180 in controls using the standard phospho-specific antibody. We only detect clear T180 phosphorylation of endogenous MST1/2 when cells are treated with the phosphatase inhibitor okadaic acid. Therefore, for this experiment we moderately overexpressed MST1 so that we could see some phosphorylation at T180 and were then able to test whether TRIP6 overexpression reduced MST1-T180 phosphorylation.

2) The problems in labeling Figure 2A have been corrected.

Reviewer #2

Specific Concerns

1) As suggested we include a model that summarizes the binding and regulatory interactions that we observe as a schematic that is part of the online version of the manuscript.

2) As suggested we now mention in the introduction other studies showing interaction between LATS1/2 and Zyxin/Ajuba. We also clarify that the two regions of LATS2 (amino acids 376-397 and 625-644) that we tested for TRIP6 binding were based on the regions of LATS1/2 previously defined as responsible for interacting with the TRIP6 family members Ajuba and Zyxin.

3) The reviewer suggests that a silver stain gel be done on LATS1/2 immunoprecipitations to assess "how stringent the conditions are, and how specific the interaction is." We think that the LATS1-TRIP6 interaction is "specific" in the sense that TRIP6 is not simply coming down with beads or antibody, since we do not observe TRIP6 in IgG control immunoprecipitations. We also do not make any claim that TRIP6 is the only protein coming down in LATS1/2 immunoprecipitations. We have essentially done this experiment when we analyzed LATS2 pull downs by mass spectrometry (Paramasivam et al., 2011). We see many LATS2 binding proteins, including TRIP6 and some other LIM domain proteins. But as we show here using recombinant proteins,TRIP6 can bind directly to LATS2, and many other experiments clearly show that TRIP6-LATS1/2 association is regulated by stimuli that affect LATS1/2 regulation. Determining the relationship between TRIP6 and other LIM domain proteins is an interesting question, but beyond the scope of the present study.

4) The reviewer is interested in whether any of the treatments or knockdowns affects cell junctions. Some of these controls were shown previously, but we have now done E-Cadherin staining in cells where TRIP6, LATS1/2, and Vinculin have been knocked down (Figure EV4C), and we do not see any obvious change in E-Cadherin staining. In addition, we test whether knockdown of TRIP6 in cells grown to high density interferes with recruitment of Vinculin to cell-cell junctions following stretch. We observe that Vinculin is recruited normally to cell-cell junctions in a tension dependent manner in TRIP6 depleted cells. This result is consistent with our other data suggesting that TRIP6 acts downstream of Vinculin and that TRIP6 depletion is not radically disrupting adherens junctions. That said, we cannot rule out that there are subtle changes in junction architecture, and we have stated this in the results and discussion.

5) We agree with the reviewer about the important distinction between "mechanosensation" and "mechanoresponse" and have now corrected the way this is used in the paper.

6) The reviewer states: "*Your assertions about what your data demonstrates in regards to the vinculin-Trip6 interaction and regulation thereof are overstated. While I agree that an inference can be made, you go beyond this in the final sentences of the Results section. High throughput Y2H and presence in the immune complex are not convincing evidence of a primary interaction*."

Response: While an interaction between vinculin and TRIP6 in the yeast 2-hybrid system is unlikely to be indirect (due bridging proteins), we agree with the reviewer that we cannot rule out the possibility that vinculin and TRIP6 interact via bridging proteins in our system. Therefore, we changed how we describe the interaction in the text, and in particular the sentences at the end of the results, as suggested, to reflect this.

Reviewer #3

1) The reviewer states: "*The authors propose that TRIP6 inhibits LATS1/2 kinases by competing with MOB for LATS binding. This is supported by a CoIP experiment in which the expression of TRIP6 has been shown to reduce LATS binding (Fig 2A) and an in vitro competition binding assay (Fig 2B). To provide more evidence for this mechanism, the authors could also investigate the effect of TRIP6 mutant construct that is deficient in LATS binding (1-277), in the LATS-MOB CoIP experiment, which presumably would not decrease LATS-MOB binding upon expression*."

Response: We have done the suggested experiment and observe that, unlike full length TRIP6, the TRIP6-(1-277) protein does not decrease LATS2-MOB1 binding. This data replaces the old Figure 2A.

2) The reviewer states: "*In Figure 5, Vinculin has been shown to interact with and recruit TRIP6 to cell-cell junctions and this is proposed as a mechanism through which tension at cell junctions regulate LATS1/2 kinases. Although TRIP6 interaction with LATS and Vinculin has been investigated separately, it is important to demonstrate concomitant binding and complex formation between TRIP6, LATS and Vinculin in a CoIP experiment to further support this model. It would also make a stronger case for the proposed mechanism to demonstrate a decrease in protein interaction between TRIP6-LATS-Vinculin in response to at least one of the conditions that reduces TRIP6-Vinculin and TRIP6-LATS binding (i.e. high cell density or loss of actin stress fiber)*."

Response: As suggested, we have immunoprecipitated LATS1 and blotted for the presence of TRIP6 and vinculin in the presence or absence of LatB or serum to disrupt F-actin and stress fibers. In both cases, we see reduced TRIP6 and vinculin in LATS1 immune complexes when F-actin and stress fibers are perturbed (see Figure 6E-F).

3) "*The authors might also look at LATS phosphorylation (S909/T1079) upon Vinculin loss of function*."

Response: We have done this experiment and observed that both LATS1/2 (S909/T1079) and YAP (S127) phosphorylation increase when vinculin is knocked down consistent with our other experiments. This result is shown in Figure 5D.

4) "*Since the authors propose a LATS-dependent model for TRIP6 mechanism of action it would be nice, though not critical, to investigate the effect of TRIP6 depletion on subcellular localization of YAP mutants that are refractory to LATS inhibition (YAP-S127 or YAP5SA)*."

Response: To test whether the effects of TRIP6 deletion (TRIP6-KO) on YAP localization depend on LATS1/2, we have knocked down LATS1/2 in TRIP6-KO cells. We see that depletion of LATS1/2 restores nuclear YAP localization in TRIP6-KO cells, consistent with TRIP6 acting through LATS1/2 to affect YAP localization.

Minor errors/comments:
Response: The figure labeling errors have been corrected. The reviewer also suggests that we not refer to TRIP6 null cells generated using CRISPR-Cas9 as "TRIP6Δ" and instead use "TRIP6-KO". We have now done this. Also, all experiments involving siRNA or shRNA are labeled as such.

2nd Editorial Decision 8 November 2017

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the referees that were asked to re-evaluate your study (you will find enclosed below). As you will see, referees #2 and #3 now support the publication of your manuscript in EMBO reports. Referee #1 has several minor suggestions to improve the paper that we ask you to address in a final revised version of the manuscript.

Further, I have the following editorial requests that also need to be addressed:

Please add a short running title and up to five keywords to the manuscript title page.

Please format the references according to EMBO reports style. See:
http://embor.embopress.org/authorguide#referencesformat

Some of the Western blot panels look over-contrasted (e.g. in Fig. 3E). Please provide all Western blot images with similar background intensities, with as little modification and contrast-adjustment compared to the original source files. An example is e.g. panel H of Fig. EV1, where the Vinculin blot seems to have been exposed very long, the tubulin loading control very short.

As all the Western blot panels have been cropped substantially, we strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of entire gels or blots) of your experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

It seems there is no legend for Fig. 4C. Please add this.

Please label the 2 appendix tables correctly. Please use the nomenclature Appendix Table Sx and also add callouts using this nomenclature in the text (the methods section, I guess).

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

REFEREE REPORTS

Referee #1:

The authors addressed most of my comments and suggestions and their additional experimentation has strengthened the paper. I think that the paper is suitable for publication.
I have few minor points/text changes regarding the figure legends.

Minor comments:
Figure 1C: The molecular weight of the 137-677 construct seems different between IP samples and lysates. Perhaps indicate the molecular weight marker bands so that readers have an idea about the size of the different constructs.
Figure 3H: What condition do the white bars represent? This is not apparent from the figure legend.
Figure EV4 B, C: "E-cadherin" is not written with a capital E in the Merged lane.

Figure legends:
In the figure legend the authors frequently refer to other figures by including the Figure number, e.g. "5D" or "EV3". To avoid confusion I would suggest to include word Figure, e.g. Figure 5D and Figure EV3.
Figure 1A, perhaps indicate for abbreviation LIM and PDZ for inexperience readers.
Figure 3G: "shControl" is written as "shEGFP" in the figure legend.
Figure 5 and EV5: In cases of the merged immunostainings "vinculin" is abbreviated as "vin" but this is not indicated in the legend.
Figure EV1, EV3, EV5: When quantifying the fluorescence at cell junctions or YAP nuclear localization, please indicate the number of cells you used for the analysis.
Figure EV3 B: I assume WT (as written in the figure legend) is control (as written in the figure). I would suggest to have an uniform description.

Referee #2:

The authors have provided thorough and thoughtful responses to the questions and issues I presented. I recommend this manuscript is ready for publication in EMBO.

Referee #3:

The authors have adequately addressed all of my concerns.

2nd Revision - authors' response 13 November 2017

We have incorporated all of the suggestions of reviewer #1, and your additional suggestions regarding contrast levels in western blot panels and other small corrections.

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: Dannel McCollum
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2017-44777V1

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be 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 $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	All western blotting experiments were done in triplicate. RT-QPCR experiments were done in triplicates. All microscopy experiments were done in triplicate with at least 100 cells counted for each experiment. Statistical methods are described in a separate section of the Materials and Methods.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All samples were included in our analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	All microscopy experiments were examined blind by someone other than the person who prepared the samples.
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	Yes

USEFUL LINKS FOR COMPLETING THIS FORM

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

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

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

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

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

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibody information is listed in the appendix table.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes. All the information is listed in the appendix table.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
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
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	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
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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