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MARK4 inhibits Hippo signalling to promote proliferation and migration of breast cancer cells

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 03 May 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 are quite in agreement and acknowledge the potential interest of the findings. However, all three referees have raised a number of concerns and suggestions to improve the manuscript, to strengthen the data and to substantiate the conclusions drawn. All five points of referee #1 are very important and should be addressed experimentally, in particular the interaction studies need to be confirmed with endogenous proteins or protein levels (which is also mentioned several times by referee #3). Also the first point of referee #3 and several of his/her other points are important. However, we do not think the roles of MARK2 and 3 need to be further addressed experimentally, as this manuscript is centered on MARK4.

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 the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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REFEREE REPORTS

Referee #1:

The MS by Attisano and colleagues presents evidence that MARK4 is an activator of YAP or TAZ activity. The data are gain of function in HEK293T and loss of function (by Crispr/Cas9 and siRNAs) in MDA MB 231, using as read-outs YAP localization and TEAD-luciferase (upon overexpression), or activation of endogenous targets (in transfected siRNA assay). This is then corroborated by Phos-TAG gels and biochemistry/CoIP suggesting that Mark4 activates YAP by inhibiting MST1/2 and Sav. The MS has some gaps, also taking into account a recent paper in NCB by Camargo and colleagues with essentially opposite conclusions (although the collective evidence of this MS appears stronger):

1) if the mechanisms is through MST1/2 and Sav, the knockdown of MST1/2 and Sav proteins should rescue the effect of the Crispr knockout clones in term of TEAD-lux, endogenous targets and localization. Is it so?

2) Fig2B is mislabeled. Irrespectively, there is no real effect of Mark4 on phosphorylation, which is at odd with the striking effects on localization, and with the model proposed.

3) The bioassay used in the last figure is cell migration, making a pure correlation between the effect of YAP or TAZ siRNAs and the similar effects (inhibition of migration) of the MARK4 KO. However, MARKs are very powerful regulators of several cellular pathways potentially involved in cell migration, besides YAP/TAZ regulation. For example, targets include microtubules, factors involved in front-rear polarity, lamellipodia etc. The best experiment to sort this out would be to challenge a phospho-mutant YAP unable to be phosphorylated by Hippo in migration and proliferation assays. This YAP active mutant should be insensitive to MARK KO (once introduced in their MARK KO cells) whereas wild-type YAP should be still sensitive and serve as control.

4) In light of the discrepancy above mentioned with the NCB, what is somehow disturbing is the fact that HEK293T cells were used in both papers. The loss of function should be re-examined in different cell lines, including those used in the NCB paper, otherwise this is quite confusing. The generality of the various claims is now not secondary for this story.

5) The biochemistry mainly relies on tagged protein CoIPs. To corroborate the conclusions, they should invest on interaction between endogenous proteins.

Referee #2:

Arash et al show a role of MARK4 in regulation of the Hippo pathway. Based on a functional screen of a TEAD-luciferase reporter, the authors found that MARK4 overexpression increased the reporter activity. MAPK4 overexpression also decreased YAP/TAZ phosphorylation as well as increased nuclear accumulation. Consistently, knockout of MARK4 decreased nuclear YAP/TAZ. Mechanistic studies indicated that MARK4 could phosphorylate both MST and SAV and disrupt the interaction between MST/SAV and LATS. MARK4 knockdown or knockout reduced cell proliferation and migration.

Identification of MARK4 as a new regulator of the Hippo signaling pathway is potentially significant. Based on the data presented, the effect of MARK4 on the phosphorylation of YAP/TAZ is rather modest, indicating that MARK4 is unlikely to be a major regulator of the Hippo pathway. A few key questions need to be answered before one can conclude MARK4 as an upstream regulator of the Hippo pathway by disrupting MST/SAV and LATS interaction.

MARK4 is a rather promiscuous kinase and overexpression can easily lead to nonspecific effect. Is

the phosphorylation status of MST and SAV altered in MARK4 knockout cells? The interaction of endogenous MST, SAV, and LATS can be readily detected and such antibodies are commercially available. The authors need to examine the interaction among these proteins by comparing MARK4 WT and knockout cells. The effect of MARK4 knockdown or knockout on cell proliferation and migration could be completely unrelated to the Hippo pathway. Experimental evidence is needed to show that the Hippo pathway is mediating the effect of MARK4 on cell proliferation and migration.

Additional comments:

1.) The identity of genes identified by the screen (Fig.1B) should be presented in the manuscript.
2.) Fig. 2B, are siYAP and siTAZ mislabeled in the figure?
3.) Fig.3A, 3B. MARK4 KO had a very minor effect on TAZ phosphorylation but a rather dramatic effect on subcellular localization. This data would suggest that MARK4 affects TAZ localization independent of TAZ phosphorylation. This possibility can be tested by expressing WT and phosphorylation defective TAZ in the MARK4 WT and KO cells.
4.) Fig. 5B. To be consistent, "+" in HA-MARK4 should be changed to "WT".
5.) Fig. 7. Why cell proliferation was not analyzed using the MARK4 KO cells? To support that MARK4 affects the Hippo pathway, expression of genes that are regulated by the Hippo pathway should be determined in the MARK4 knockdown or KO cells.
6.) Mechanistically, the study would be significantly enhanced if the authors can identify the MARK4-induced phosphorylation sites in MST and/or SAV responsible for weakening their interaction with LATS.

Referee #3:

Arash et al. attempt in this manuscript to mechanistically link MARK kinase signalling with the Hippo pathway in the context of cancer properties of human breast cancer cells. While this manuscript has the potential to provide novel and interesting leads, it is unfortunately rather a yet incomplete study in its current form. Considering that MARK kinases have already been linked to Hippo signalling (see point 10 below), it would be really important to provide sufficient mechanistic insights in this manuscript in order to sufficiently support the novelty of this study. Overall, I consider the manuscript in its current form too preliminary to fully support the big statements made throughout this manuscript.

Major points:

1) Evidence to support statement in abstract is missing
In the abstract the authors state that "MARK4 acts as negative regulator of the Hippo kinase cassette to promote YAP/TAZ activity". Where do the authors show that MST1/2 and LATS1/2 kinase activities are changed upon MARK4 manipulations? Where is the evidence showing that YAP/TAZ phosphorylation on key regulatory sites such as Ser127 and Ser381 (also known as Ser397) is altered by MARK4 manipulations?

2) Define effects on the cytoskeleton in the context of MARK4 manipulations.
Considering that MARKs can play roles in the cytoskeleton and that cytoskeleton re-arrangements can affect Hippo signalling, the authors should define the level of changes to the actin/tubulin cytoskeleton upon MARK4 manipulations. For example, does the level of YAP/TAZ nuclear localisation correlate with cytoskeleton changes?

3) Consistency of findings regarding MARK2/3/4: In Figure 1C, the reader learns that MARK2, MARK3 and MARK4 overexpression has an effect on the TEAD luciferase reporter readout. Then we are told that only MARK4 knockdown has an effect on YAP/TAZ target genes (Fig EV1). Then in Figure 4D, MARK2 and MARK3 are studied again, showing that they can phosphorylate MST2 like MARK4. So, how does this then work at the end? According to the interpretation of the authors, the regulation of MST2 phosphorylation by MARK kinases is inhibitory of Hippo core signalling. However, why are then the findings regarding MARK2 and MARK3 not consistent with the data on MARK4 ?

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4) Provide more substantial data for Figure 2:
(i) The phosphorylation status of YAP should be studied using commercially available antibodies that work very nicely for immunoblotting (i.e. anti-Ser127-P and anti-Ser381/397-P from Cell Signaling).
(ii) The IF pictures shown in Fig. 2A and 2C should be backed up by quantifications.
(iii) The IF pictures shown in Fig. 2A and 2C need to be complemented with biochemical fractionation experiments (i.e. nuclear vs. cytoplasmic fractions).

5) Provide more substantial data on Figure 3:
(i) Study the phosphorylation status of YAP using phospho-specific antibodies.
(ii) Back up the IF pictures shown in Fig. 3B with quantifications.
(iii) Complement IF pictures shown in Fig. 3B with biochemical fractionation experiments.
(iv) Expand Fig. 3C and 3D with MARK2 and MARK3. Does the overexpression of MARK2/3 have the same effect as MARK4?
(v) And most importantly regarding Figure 3, if the model of the authors is correct, then the shown MARK4 manipulations should affect the expression of established YAP/TAZ target genes. Is this the case here. In regard to the data shown in Figures 1 to 3, the authors state on page 7 that "... provide compelling evidence ... confirm the requirement for MARK4 kinase activity for Hippo pathway regulation." I hope that the authors have realised based on my feedback that they are lacking substantial evidence regarding the regulation of the Hippo pathway in Figures 2 and 3. Without expanding the experiments as outlined above, this is not compelling evidence.

6) Provide substantial extensions for Figure 4:
(i) Show endogenous interaction of MARK4 with MST2.
(ii) Probe for T-loop phosphorylation of MST1/2 in the MARK4 kinase assay.
(iii) Perform kinase assay with radiolabelled ATP (still the golden standard).
(iv) Does MARK4 change the kinase activity of MST1/2 kinases? (monitor a well-established MST1/2 substrate such as MOB1)

7) Provide substantial extensions for Figure 5:
(i) Show complex formation on the endogenous level. (Currently all the data shown in Figures 4, 5 and 6 are solely based on overexpression experiments.)
(ii) Perform kinase assay with radiolabelled ATP (still the golden standard).

8) Provide substantial extensions of Figure 6:
(i) Show complex formation on endogenous level.
(ii) Study T-loop phosphorylation of MST1/2. (phospho antibodies and T-loop mutants)
(iii) Study the phosphorylation of LATS1/2.

9) Provide substantial extensions of Figure 7:
(i) Study proliferation of knockout cells shown in Figure 3A. Does this match Fig. 7A?
(ii) Study YAP knockdown in Figure 7A.
(iii) Does the expression of Hippo-insensitive YAP-5SA rescue the effect of siMARK4 in Figure 7A?
(iv) Confirm that the effects shown in 7A and 7C are Hippo core kinase dependent. Does MST1/2 and/or LATS1/2 overexpression mimic YAP/TAZ depletion?

10) Redundancy with refs. 46, 47, and 48?
As the authors point out themselves, it has already been reported that MARK kinases are linked to the Hippo pathway. Therefore, it is very important to provide a clear mechanistic understanding in this manuscript in order to make a significant step forwarded. Otherwise, this study is at risk to be categorised as another study that shows that MARK kinases can be linked to Hippo signalling.

Minor points:

A) Clarify signalling in Hippo core cassette

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In the abstract on page 2 and in the introduction section on page 3, the authors need to spell out very clearly for the non-expert that MST1/2 (Hippo) kinases are responsible for the activation of LATS1/2 (Warts) kinases and that then activated LATS1/2 (Warts) is inactivating YAP/TAZ through the phosphorylation of different regulatory sites on YAP/TAZ. Right now the description of this point is not clear enough.
B) Include reference for RASSFs in Hippo signalling on page 3.
C) Include references from the Pan laboratory on page 4 together with refs. 23-26.
D) Include table to summarise hits shown in Figure 1B.
In order to more appreciate the findings of the screen presented in Figure 1A/B, the authors maybe want to consider to include a table summarising the hits and their quantification.
E) Labelling of Figure 7.
Please make sure to label TAZ throughout since the non-expert might not know what WWTR1 actually is.
F) Re-phrase text regarding SIK citation (ref. 36).
The authors should make it very clear that the molecular mechanisms described in ref. 36 cannot be conserved between flies and humans, since the phosphorylation site on fly Salvador does not exist in human Salvador. This needs to be made very clear for the non-expert reader.

1st Revision - authors' response 19 September 2016

Response to Referees' Comments

In this revised manuscript, we have incorporated the referee's helpful comments and suggestions, all of which have greatly enhanced our manuscript. Two major additions include: ***1)*** new data using siRNAs and/or overexpression of a phosphorylation site mutant of TAZ to demonstrate that MARK4 functions through the hippo kinase cassette to regulate YAP/TAZ localization, activity and cellular responses (growth and migration) and ***2)*** demonstration of endogenous interactions among MARKs, MST and SAV. This additional data along with the incorporation of many other helpful referee suggestions into our manuscript, have significantly strengthened our conclusions that MARK4 inhibits the activity of the Hippo kinase cassette and thereby promotes YAP/TAZ activity and tumorigenic-like responses in cells.

Referee #1:

The MS by Attisano and colleagues presents evidence that MARK4 is an activator of YAP or TAZ activity. The data are gain of function in HEK293T and loss of function (by Crispr/Cas9 and siRNAs) in MDA MB 231, using as read-outs YAP localization and TEAD-luciferase (upon overexpression), or activation of endogenous targets (in transfected siRNA assay). This is then corroborated by Phos-TAG gels and biochemistry/CoIP suggesting that Mark4 activates YAP by inhibiting MST1/2 and Sav. The MS has some gaps, also taking into account a recent paper in NCB by Camargo and colleagues with essentially opposite conclusions (although the collective evidence of this MS appears stronger):

1) if the mechanisms is through MST1/2 and Sav, the knockdown of MST1/2 and Sav proteins should rescue the effect of the Crispr knockout clones in term of TEAD-lux, endogenous targets and localization. Is it so?

We concur that this is an important point. Thus to demonstrate that MARK4 acts through MST and SAV, we abrogated MST and SAV expression in MARK4 CRISPR knockout (KO) cells using specific siRNAs and analyzed YAP/TAZ localization and target gene expression by IF microscopy and qPCR, respectively. As shown in Fig 3C and D, loss of expression of MST2 or SAV1 rescued the nuclear localization of YAP/TAZ and restored ANKRD1 expression in MARK4 KO clones. Analogously, we tested the effect of siMST2 and siSAV1 in siMARK4 transfected cells and also confirmed that ANKRD1 expression was rescued (Fig 3E).

2) Fig2B is mislabeled. Irrespectively, there is no real effect of Mark4 on phosphorylation, which is at odd with the striking effects on localization, and with the model proposed.

Once phosphorylated, YAP and TAZ are targeted for degradation, thus while the absolute level of phosphorylation does not appear to be dramatically altered in the blots, the relative ratio of phosphorylated to unphosphorylated YAP or TAZ is indeed increased as expected (Fig 2F). We neglected to clearly present this well-established feature of YAP/TAZ phosphorylation and have now modified the text accordingly. In addition, a plot showing quantitation of phosphorylated to non-phosphorylated YAP and TAZ has been added (Fig. 2F). We have also added a new experiment in which YAP phosphorylation was assessed using a phospho-YAP(S127) specific antibody (Fig 2G), rather than PhosTag gels. Quantitation of this blot also shows that there is an increase in the relative level of phosphorylated to total YAP upon siMARK4 knockdown. Of note, in this experiment cells were lysed 48 h after transfection (versus 24 h in Fig 2F) so that the expected reduction in overall YAP/TAZ levels is more evident.

In further support of our conclusions that phosphorylation is important and as suggested by referee #2, we also generated MDA-MB-231 cells expressing either a wild-type or phosphorylation site mutant (S89A) of TAZ and evaluated TAZ localization upon MARK4 knockdown. As shown in Fig 3A, MARK4 knockdown in TAZ WT cells resulted in loss of nuclear localization or complete loss of staining due to TAZ degradation while TAZ S89A expressing cells show a predominantly nuclear TAZ localization in both siControl and siMARK4 transfected cells.

Finally, we have corrected the mis-labelling of the siYAP/siTAZ lanes.

3) The bioassay used in the last figure is cell migration, making a pure correlation between the effect of YAP or TAZ siRNAs and the similar effects (inhibition of migration) of the MARK4 KO. However, MARKs are very powerful regulators of several cellular pathways potentially involved in cell migration, besides YAP/TAZ regulation. For example, targets include microtubules, factors involved in front-rear polarity, lamellipodia etc. The best experiment to sort this out would be to challenge a phospho-mutant YAP unable to be phosphorylated by Hippo in migration and proliferation assays. This YAP active mutant should be insensitive to MARK KO (once introduced in their MARK KO cells) whereas wild-type YAP should be still sensitive and serve as control.

We generated both MDA-MB-231 and MCF10A cells stably overexpressing phospho-site mutants of YAP (5SA) and/or TAZ (S89A), and in the case of MDA-MB-231 TAZ S89A, used them successfully to show a requirement for MST/SAV in MARK4-regulated localization of YAP/TAZ and target genes (Fig 3A, see response to comment 2 above). However, we found that constitutive expression of the mutant YAP or TAZ variants resulted a pronounced decrease in the rate of cell growth and migration that made it challenging to reliably compare the effect of siMARK4 on cells expressing wild-type or mutant YAP and TAZ. Thus, as an alternative to address this question, we used a siRNA approach and now show that loss of MST2 or SAV1 rescues the ability of siMARK4 to decrease cell migration and growth, supporting our hypothesis that the effects of siMARK4 are through regulation of the Hippo pathway.

4) In light of the discrepancy above mentioned with the NCB, what is somehow disturbing is the fact that HEK293T cells were used in both papers. The loss of function should be re-examined in different cell lines, including those used in the NCB paper, otherwise this is quite confusing. The generality of the various claims is now not secondary for this story.

As per the referee's suggestion, we abrogated the expression of MARK4 in three additional cell lines including another breast cancer cell line, MCF10A, and two colorectal cancer cell lines, namely SW480 and DLD-1, the later of which was also used in the NCB paper. Consistent with our data in MDA-MB-231 cells, loss of MARK4 results in a decrease in YAP/TAZ target gene expression (Fig 1E).

5) The biochemistry mainly relies on tagged protein CoIPs. To corroborate the conclusions, they should invest on interaction between endogenous proteins.

We now provide additional data (Fig 5C, 5D, 6E and 6F) that demonstrate interactions among endogenous proteins. Specifically, we first performed a semi-endogenous co-immunoprecipitation (CoIP) experiment by expressing Flag-MARK4 in HEK293T cells and show association of Flag-MARK4 with endogenous SAV1 and MST (Fig 5C). Demonstrating interactions with endogenous MARK4 proved intractable due to the poor quality of available MARK4 antibodies. However, given

that MARK3 can activate the Hippo TEAD-luc reporter (Fig 1C) and that highly specific antibodies are available, we focused further effort on MARK3. We found that similar to siMARK4 in MDA-MB-231 cells, abrogation of MARK3 expression using siRNAs in MDA-MB-468 cells, resulted in increased YAP/TAZ phosphorylation and decreased YAP/TAZ target gene expression (Fig EV2). Next, by immunoprecipitating MST and/or SAV in either MDA-MB-468 or HEK293T cells, we showed physical interactions of endogenous MARK3 with MST1 and SAV1 (Fig. 5D, 6E and F).

Referee #2:

Arash et al show a role of MARK4 in regulation of the Hippo pathway. Based on a functional screen of a TEAD-luciferase reporter, the authors found that MARK4 overexpression increased the reporter activity. MAPK4 overexpression also decreased YAP/TAZ phosphorylation as well as increased nuclear accumulation. Consistently, knockout of MARK4 decreased nuclear YAP/TAZ. Mechanistic studies indicated that MARK4 could phosphorylate both MST and SAV and disrupt the interaction between MST/SAV and LATS. MARK4 knockdown or knockout reduced cell proliferation and migration. Identification of MARK4 as a new regulator of the Hippo signaling pathway is potentially significant. Based on the data presented, the effect of MARK4 on the phosphorylation of YAP/TAZ is rather modest, indicating that MARK4 is unlikely to be a major regulator of the Hippo pathway. A few key questions need to be answered before one can conclude MARK4 as an upstream regulator of the Hippo pathway by disrupting MST/SAV and LATS interaction. MARK4 is a rather promiscuous kinase and overexpression can easily lead to nonspecific effect. Is the phosphorylation status of MST and SAV altered in MARK4 knockout cells?

We attempted to detect phosphorylation of endogenous MST and SAV by immunoblotting using PhosTag gels but were unable to observe any upshifted (ie phosphorylated) bands for either protein in untreated cells, thus it was not possible to show that loss of MARK4 decreased phosphorylation. We speculate that only a small proportion of endogenous MST and/or SAV is phosphorylated and while undetectable with the currently available antibodies, this is sufficient to inactivate Hippo.

The interaction of endogenous MST, SAV, and LATS can be readily detected and such antibodies are commercially available. The authors need to examine the interaction among these proteins by comparing MARK4 WT and knockout cells. The effect of MARK4 knockdown or knockout on cell proliferation and migration could be completely unrelated to the Hippo pathway. Experimental evidence is needed to show that the Hippo pathway is mediating the effect of MARK4 on cell proliferation and migration.

We concur these are important points. As detailed above in response to referee #1, we now provide new data (Fig 5C, 5D, 6E and 6F) that demonstrate interactions among endogenous proteins. In addition, we have added new data to show that the effects of MARK4 on cell growth and migration are dependent on the Hippo pathway. Specifically, we evaluated the effect of concomitant abrogation of MST or SAV expression in siMARK4 transfected cells and as shown in Fig 8F and 8G, observed that knockdown of MST or SAV effectively rescues the decrease in cell growth and migration observed upon MARK4 depletion.

Additional comments:

The identity of genes identified by the screen (Fig.1B) should be presented in the manuscript.

The results from the screens have not been subjected to detailed statistical analysis or verification that would give meaningful confidence levels for identified regulators. Thus, we prefer not to include the data, as is. In light of this, if it is felt to be appropriate, we can remove the plot (Fig 1B) showing the summary of the screen results and simply state that we identified MARKs in a siRNA screen.

Fig. 2B, are siYAP and siTAZ mislabeled in the figure?

Thanks for pointing this out. The labeling has been corrected in the revised version.

Fig.3A, 3B. MARK4 KO had a very minor effect on TAZ phosphorylation but a rather dramatic effect on subcellular localization. This data would suggest that MARK4 affects TAZ localization independent of TAZ phosphorylation. This possibility can be tested by expressing WT and phosphorylation defective TAZ in the MARK4 WT and KO cells.

As detailed above in response to similar comments by referee #1, once phosphorylated, YAP and TAZ are targeted for degradation, thus while the absolute level of phosphorylation does not appear to be dramatically altered in the blots, the relative ratio of phosphorylated to unphosphorylated YAP or TAZ is indeed increased as expected (Fig 2F). We modified the text to present this information more clearly and have added a plot showing quantitation of phosphorylated to non-phosphorylated YAP and TAZ. We also added a panel (Fig 2G) in which quantitation of YAP phosphorylation, assessed using a phospho-YAP(S127) specific antibody also shows that there is an increase in the relative level of phosphorylated to total YAP upon siMARK4 knockdown. Of note, in this experiment cells were lysed 48 h after transfection (versus 24 h in Fig 2F) so that the expected reduction in overall YAP/TAZ levels is more evident.

Finally, in further support of our conclusions and as suggested by this referee, we also generated MDA-MB-231 cells expressing either wild-type or a phosphorylation site mutant (S89A) of TAZ and evaluated TAZ localization upon MARK4 knockdown. As shown in Fig 3A, MARK4 knockdown in TAZ WT cells resulted in loss of nuclear localization or complete loss of staining due to TAZ degradation while TAZ-S89A expressing cells show a predominantly nuclear TAZ localization in both siControl and siMARK4 transfected cells.

Fig. 5B. To be consistent, "+" in HA-MARK4 should be changed to "WT".

It has been changed to WT as suggested.

Fig. 7. Why cell proliferation was not analyzed using the MARK4 KO cells? To support that MARK4 affects the Hippo pathway, expression of genes that are regulated by the Hippo pathway should be determined in the MARK4 knockdown or KO cells.

As suggested, we evaluated cell growth and target gene expression in the MARK4 knockout (KO) cells and now show that similar to siRNA-mediated knockdown, there is a decrease in cell growth (Fig 8H) and target gene expression (Fig EV3D), in MARK4 KO cells versus controls.

Mechanistically, the study would be significantly enhanced if the authors can identify the MARK4-induced phosphorylation sites in MST and/or SAV responsible for weakening their interaction with LATS.

While identifying the sites and developing phospho-specific antibodies would further enhance our study, despite our attempts using mass spectroscopy and site directed mutagenesis, we have not yet succeeded in this, thus, this will have to be part of future studies.

Referee #3:

Arash et al. attempt in this manuscript to mechanistically link MARK kinase signalling with the Hippo pathway in the context of cancer properties of human breast cancer cells. While this manuscript has the potential to provide novel and interesting leads, it is unfortunately rather a yet incomplete study in its current form. Considering that MARK kinases have already been linked to Hippo signalling (see point 10 below), it would be really important to provide sufficient mechanistic insights in this manuscript in order to sufficiently support the novelty of this study. Overall, I consider the manuscript in its current form too preliminary to fully support the big statements made throughout this manuscript.

Major points:

1) Evidence to support statement in abstract is missing: In the abstract the authors state that "MARK4 acts as negative regulator of the Hippo kinase cassette to promote YAP/TAZ activity". Where do the authors show that MST1/2 and LATS1/2 kinase activities are changed upon MARK4

manipulations? Where is the evidence showing that YAP/TAZ phosphorylation on key regulatory sites such as Ser127 and Ser381 (also known as Ser397) is altered by MARK4 manipulations?

As detailed above and below in response to specific referee comments, we now provide several additional lines of evidence to support our statement that MARK4 functions by inhibiting the kinase cassette. In brief, this includes our demonstration that loss of the kinase cassette components, MST and/or SAV rescues the effect of siMARK4 on YAP/TAZ localization, target gene expression, cell growth and migration (Fig 3D, E, 8F and G), and that siMARK4 does not alter localization of a phosphorylation site mutant of TAZ (Fig 3A).

2) Define effects on the cytoskeleton in the context of MARK4 manipulations: Considering that MARKs can play roles in the cytoskeleton and that cytoskeleton re-arrangements can affect Hippo signalling, the authors should define the level of changes to the actin/tubulin cytoskeleton upon MARK4 manipulations. For example, does the level of YAP/TAZ nuclear localisation correlate with cytoskeleton changes?

While changes in the microtubule network have not yet been linked to the Hippo pathway, alterations in the actin cytoskeleton have a well-established role in regulating YAP/TAZ activity. Whether the Hippo kinase cassette is involved in this, is a subject of current controversy. While understanding how MARKs function in the context of mechanotransduction to regulate YAP/TAZ activity is certainly an interesting area of investigation, we feel that undertaking a detailed analysis of this question is beyond the scope of the current manuscript. We have added a sentence in the discussion to indicate this area could be pursued in future studies.

3) Consistency of findings regarding MARK2/3/4: In Figure 1C, the reader learns that MARK2, MARK3 and MARK4 overexpression has an effect on the TEAD luciferase reporter readout. Then we are told that only MARK4 knockdown has an effect on YAP/TAZ target genes (Fig EV1). Then in Figure 4D, MARK2 and MARK3 are studied again, showing that they can phosphorylate MST2 like MARK4. So, how does this then work at the end? According to the interpretation of the authors, the regulation of MST2 phosphorylation by MARK kinases is inhibitory of Hippo core signalling. However, why are then the findings regarding MARK2 and MARK3 not consistent with the data on MARK4?

When overexpressed, all three MARKs can activate the TEAD-luc reporter and can phosphorylate MST2, suggesting that they could all have similar functions in regulating Hippo. In this study, we focused most of our subsequent analyses on one family member, MARK4, as siRNA-mediated depletion of MARK4 showed the strongest effect on YAP/TAZ in MDA-MB-231 cells. In the revised manuscript, we have now added new data showing that loss of MARK3 in a different cell line, MDA-MB-468, increases YAP phosphorylation (pS127) and decreases YAP/TAZ target gene expression (Fig EV2). Thus, the MARK family member that is most pertinent for regulating the Hippo pathway varies depending on the cell line. This context-dependent role for MARKs is now mentioned in the text.

4) Provide more substantial data for Figure 2:
(i) The phosphorylation status of YAP should be studied using commercially available antibodies that work very nicely for immunoblotting (i.e. anti-Ser127-P and anti-Ser381/397-P from Cell Signaling).

As requested, we have added a panel (Fig 2G) in which YAP phosphorylation was assessed using the phospho-YAP (S127) antibody. This antibody does not recognize endogenous phospho-TAZ, thus we continued to use only PhosTag gels to visualize TAZ phosphorylation.

(ii) The IF pictures shown in Fig. 2A and 2C should be backed up by quantifications.

Images have been quantitated and plots are now included in the manuscript.

(iii) The IF pictures shown in Fig. 2A and 2C need to be complemented with biochemical fractionation experiments (i.e. nuclear vs. cytoplasmic fractions).

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We believe that IF experiments are a sufficient and a more reliable method to evaluate the subcellular localization of YAP/TAZ as it avoids concerns of alterations in protein localization that could occur during cell fractionation procedures.

5) Provide more substantial data on Figure 3.
(i) Study the phosphorylation status of YAP using phospho-specific antibodies.

See above in response to point #4i.

(ii) Back up the IF pictures shown in Fig. 3B with quantifications.

Images have been quantitated and plots are included in the manuscript.

(iii) Complement IF pictures shown in Fig. 3B with biochemical fractionation experiments.

See above in response to point #4iii.

(iv) Expand Fig. 3C and 3D with MARK2 and MARK3. Does the overexpression of MARK2/3 have the same effect as MARK4?

The main objective of this experiment was to show a rescue of the defect caused by knocking out MARK4 by re-expressing the abrogated gene. In Figure 1, we showed that overexpression of MARK2 and MARK3 activates the TEAD-luciferase reporter, and feel this is sufficient to make the point that other MARKs may also function in a manner similar to MARK4.

(v) And most importantly regarding Figure 3, if the model of the authors is correct, then the shown MARK4 manipulations should affect the expression of established YAP/TAZ target genes. Is this the case here.

In Fig 3 (now Fig. 4A), the MARK4 constructs were introduced by transient transfection, which has a very low efficiency and thus does not permit testing of the rescue effect on endogenous target gene expression. However, we did show that overexpression enhances the activity of the TEAD-luciferase reporter (Fig 1) which is considered a reliable reporter of YAP/TAZ activity.

In regard to the data shown in Figures 1 to 3, the authors state on page 7 that "... provide compelling evidence ... confirm the requirement for MARK4 kinase activity for Hippo pathway regulation." I hope that the authors have realised based on my feedback that they are lacking substantial evidence regarding the regulation of the Hippo pathway in Figures 2 and 3. Without expanding the experiments as outlined above, this is not compelling evidence.

As described above, we feel that the new additional data we have provided in response to the referee's comments does provide compelling evidence.

6) Provide substantial extensions for Figure 4.
(i) Show endogenous interaction of MARK4 with MST2.

As described above in response to Referee #1, point 5, we now provide additional data (Fig 5C, 5D, 6E and 6F) that demonstrate interactions among endogenous proteins.

(ii) Probe for T-loop phosphorylation of MST1/2 in the MARK4 kinase assay.

As requested, we probed for T-loop phosphorylation of MST1/2 in the MARK4 kinase assay using phospho-MST1/2 (T180/183) antibodies. Consistent with our model that MARK4 does not directly alter MST kinase activity, we did not observed any changes in MST autophosphorylation activity in the presence of purified MARK4 protein (Fig EV4C)

(iii) Perform kinase assay with radiolabelled ATP (still the golden standard).

Kinase assays using radiolabelled ATP have been extensively used in the past and could still be used. However, here we employed PhosTag gels, which have the advantage of permitting an

assessment of stoichiometry by comparing the proportion of phosphorylated to non-phosphorylated versions of the target protein. In this manner, it is possible to confirm that a substantial portion of the protein of interest is being phosphorylated, something that is not possible using assays with radioactive ATP.

(iv) Does MARK4 change the kinase activity of MST1/2 kinases? (monitor a well-established MST1/2 substrate such as MOB1)

MARK4 does not change the kinase activity of MST as assessed by monitoring SAV phosphorylation, also a well-established MST substrate (Fig 6A and B). Consistent with this, no changes were observed when probing MST autophosphorylation activity by blotting for T180/183 phosphorylation in an in vitro kinase assay (Fig EV4C, see response to point 6ii).

7) Provide substantial extensions for Figure 5.
(i) Show complex formation on the endogenous level. (Currently all the data shown in Figures 4, 5 and 6 are solely based on overexpression experiments.)
(ii) Perform kinase assay with radiolabelled ATP (still the golden standard).

Please see above response to point #6i and 6iii.

8) Provide substantial extensions of Figure 6.
(i) Show complex formation on endogenous level.
(ii) Study T-loop phosphorylation of MST1/2. (phospho antibodies and T-loop mutants)
(iii) Study the phosphorylation of LATS1/2.

Please see above response to point #6i and 6iii.

9) Provide substantial extensions of Figure 7.
(i) Study proliferation of knockout cells shown in Figure 3A. Does this match Fig. 7A?

As suggested, we evaluated cell growth in MARK4 KO cells and similar to our analysis using siRNA-mediated knockdown, we observed a decrease in cell growth in knockout cells compared to controls (Fig 8H).

(ii) Study YAP knockdown in Figure 7A.

We previously published that both siYAP and siTAZ decrease cell growth in MDA-MB-231 cells using the same assay (Supplementary Fig. 3 in Heidary Arash et al, (2014) EMBO J **33:** 2997-3011). We have added a citation to this study in the text.

(iii) Does the expression of Hippo-insensitive YAP-5SA rescue the effect of siMARK4 in Figure 7A?
(iv) Confirm that the effects shown in 7A and 7C are Hippo core kinase dependent. Does MST1/2 and/or LATS1/2 overexpression mimic YAP/TAZ depletion?

As described above in response to Referee #1, MDA-MB-231 and MCF10A cells stably overexpressing phospho-site mutants of YAP (5SA) and/or TAZ (S89A) displayed a pronounced decrease in cell growth, which prevented a reliable analysis of MARK4 knockdown on cells expressing wild-type or mutant YAP and TAZ. However, as an alternative to establish whether MARKs signal through the hippo cassette, we used a siRNA approach and showed that loss of MST2 or SAV1 rescued the ability of siMARK4 to decrease cell migration and growth (Fig. 8F and 8G), supporting our hypothesis that the effects of siMARK4 are through regulation of the Hippo pathway.

10) Redundancy with refs. 46, 47 and 48 ?
As the authors point out themselves, it has already been reported that MARK kinases are linked to the Hippo pathway. Therefore, it is very important to provide a clear mechanistic understanding in this manuscript in order to make a significant step forwarded. Otherwise, this study is at risk to be categorised as another study that shows that MARK kinases can be linked to Hippo signalling.

In our revised manuscript, we have provided substantial additional evidence that MARKs act by inhibiting the hippo kinase cassette. We feel the mechanistic understanding provided by our work advances the understanding of the roles of MARKs far beyond what has previously been published.

Minor points:

A) Clarify signalling in Hippo core cassette
In the abstract on page 2 and in the introduction section on page 3, the authors need to spell out very clearly for the non-expert that MST1/2 (Hippo) kinases are responsible for the activation of LATS1/2 (Warts) kinases and that then activated LATS1/2 (Warts) is inactivating YAP/TAZ through the phosphorylation of different regulatory sites on YAP/TAZ. Right now the description of this point is not clear enough.

The abstract and introductory text was re-worded to better convey this point.

B) Include reference for RASSFs in Hippo signalling on page 3.

This has been added.

C) Include references from the Pan laboratory on page 4 together with refs. 23-26.

Two references from the Pan laboratory have been added.

D) Include table to summarise hits shown in Figure 1B.
In order to more appreciate the findings of the screen presented in Figure 1A/B, the authors maybe want to consider to include a table summarising the hits and their quantification.

As we have not yet done a thorough statistical analysis of the runs including confidence scores for the hits, we prefer not to include a table of results. If it is felt to be appropriate, we can remove the plot (Fig 1B) showing the summary of the screen results and simply state that we identified MARKs in a siRNA screen.

E) Labelling of Figure 7.
Please make sure to label TAZ throughout since the non-expert might not know what WWTR1 actually is.

We changed all occurrences of WWTR1 to TAZ.

F) Re-phrase text regarding SIK citation (ref. 36).
The authors should make it very clear that the molecular mechanisms described in ref. 36 cannot be conserved between flies and humans, since the phosphorylation site on fly Salvador does not exist in human Salvador. This needs to be made very clear for the non-expert reader.

We have added text to this effect.

2nd Editorial Decision 28 September 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, referee #1 supports publication of your manuscript in EMBO reports and finds that his concerns have been adequately addressed. However, referees #2 and #3 still have concerns and think that the manuscript needs further revision. After discussing with the referees, we feel that the concerns of referees #2 and #3 need to be addressed experimentally or by additional data in a further revised version.

Regarding data quantification and statistics, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information must be provided also as short section in

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the methods part. Please provide appropriate statistics for all panels applicable.

Further, please include scale bars in all microscopy images. We also noticed thin lines in some of the panels of Fig. 2A. Please indicate what these are (or remove them).

Please upload the EV figures as single high-resolution files without legend.

It is our policy at EMBO reports have that submitted manuscripts need to be accepted within 6 months of the initial decision, which in your case would be the 3rd of December 2016. Otherwise novelty would need to be re-assessed. Presently novelty seems not to be impacted, but depending on when you submit the very final version, I might need to re-assess this again.

REFEREE REPORTS

Referee #1:

The response is adequate at addressing my concerns.

Referee #2:

The authors have addressed the majority but not all my concerns, particularly the first major point. This may be limited by the phosphoMST and phosphoSav antibodies. On the other hand, the explanation of not presenting the data/genes in original Fig.1B) is not acceptable. Such information could be valuable to the scientific community and should be included.

Referee #3:

Arash et al. have improved their manuscript to provide more evidence for a mechanistic link between MARK kinase signalling and the Hippo pathway in the context of cancer properties of human breast cancer cells. While I fully acknowledge that the authors have fully addressed all my minor comments and some of my major concerns, I do not agree with their approach to not address some of my key concerns experimentally. I still believe that it would be really important to provide sufficient mechanistic insights in this manuscript in order to sufficiently support the novelty of this study in the context of the already published links between MARK kinases and Hippo signalling (see point 10 in the detailed response to the authors). Overall, I still consider the manuscript in its revised form too preliminary to fully support the big statements made throughout this manuscript. In my opinion, the following points still have to addressed experimentally: 1, 4i, 4iii, 5i, 6ii, 6iv, and 8iii.

<u>Major points: (please see also my detailed response to the first manuscript version):</u>

1) Evidence to support statement in abstract is missing: The authors have addressed some of my concerns regarding this point. However, the kinase activities of LATS1/2 and Ser381 (Ser397) phosphorylation of YAP has not been addressed without any explanation by the authors. Minor point: please correct the labelling mistake in Figure 8F ("groth" should be "growth").

2) Define effects on the cytoskeleton in the context of MARK4 manipulations: I accept the argumentation by authors for not addressing this point experimentally, although it would have been really nice to at least see whether actin remodelling could be playing a role.

3) Consistency of findings regarding MARK2/3/4: Sufficiently addressed by the authors.

4) Provide more substantial data for Figure 2:
(i) The phosphorylation status of YAP should be studied using commercially available antibodies that work very nicely for immunoblotting (i.e. anti-Ser127-P and anti-Ser381/397-P from Cell

Signaling): Not sufficiently addressed - S381/397 phosphorylation was not tested, although the commercially available antibody works really nice.
(ii) The IF pictures shown in Fig. 2A and 2C should be backed up by quantifications: Sufficiently addressed.
(iii) The IF pictures shown in Fig. 2A and 2C need to be complemented with biochemical fractionation experiments (i.e. nuclear vs. cytoplasmic fractions): Not addressed experimentally. I disagree with the authors on this point. At least one of the IF experiments should be backed up by a completely different methodology.
5) Provide more substantial data on Figure 3:
(i) Study the phosphorylation status of YAP using phospho-specific antibodies: Not addressed. Maybe I need to be more clear. What is the phosphorylation status of endogenous YAP in MARK4 knockout cells? This is an important point to back up all the other experimental evidence.
(ii) Back up the IF pictures shown in Fig. 3B with quantifications: Sufficiently addressed.
(iii) Complement IF pictures shown in Fig. 3B with biochemical fractionation experiments: See comments above in 4iii.
(iv) Expand Fig. 3C and 3D with MARK2 and MARK3. Does the overexpression of MARK2/3 have the same effect as MARK4?: Sufficiently addressed.
(v) And most importantly, regarding Figure 3, if the model of the authors is correct, then the shown MARK4 manipulations should affect the expression of established YAP/TAZ target genes. Is this the case here?: Not addressed, but I fully accept the technical limitations here. So I do not see any reason why I should insist on this very challenging experiment. Would have been nice, but sometimes it is very tricky. Overall, the provided response is sufficient. In regard to the data shown in Figures 1 to 3, the authors state on page 7 that "... provide compelling evidence ... confirm the requirement for MARK4 kinase activity for Hippo pathway regulation." I hope that the authors have realised based on my feedback that they are lacking substantial evidence regarding the regulation of the Hippo pathway in Figures 2 and 3. Without expanding the experiments as outlined above, this is not compelling evidence.

6) Provide substantial extensions for Figure 4.
(i) Show endogenous interaction of MARK4 with MST2: Addressed sufficiently.
(ii) Probe for T-loop phosphorylation of MST1/2 in the MARK4 kinase assay: Experiment was performed, but now I am wondering why the authors detect T-loop phosphorylation of kinase-dead MST2. Could it be that they co-IP endogenous wild-type MST2? If yes, then how can we draw conclusions from this experiment? If no, how do you explain this result?
(iii) Perform kinase assay with radiolabelled ATP (still the golden standard): The response is sufficient, although it would have been nice to also include this.
(iv) Does MARK4 change the kinase activity of MST1/2 kinases? (monitor a well-established MST1/2 substrate such as MOB1): Here I also do not agree with the authors about the chosen approach. Based on which literature do the authors state that Sav is a well-established substrate of MST1/2? Based on Callus et al. (2006)? What are the phosphorylation sites on Sav? I know that many reviews write as if the Sav phosphorylation by Hippo is written in stone, but I am not aware of really solid evidence for mammalian (human Sav) as a relevant MST1/2 substrate. On the other hand, the phosphorylation of MOB1 by MST1/2 on Thr12 and Thr35 has been observed and studied by quite a few different laboratories, AND the commercially available antibodies from Cell Signaling work excellently for immunoblotting. In other words, I do not think that studying Sav phosphorylation is the way to go here.

7) Provide substantial extensions for Figure 5.
(i) Show complex formation on the endogenous level. (Currently all the data shown in Figures 4, 5 and 6 are solely based on overexpression experiments.): Sufficiently addressed
(ii) Perform kinase assay with radiolabelled ATP (still the golden standard): Sufficiently addressed.

8) Provide substantial extensions of Figure 6:
(i) Show complex formation on endogenous level: Sufficiently addressed.
(ii) Study T-loop phosphorylation of MST1/2. (phospho antibodies and T-loop mutants)
T-loop mutants have not been addressed without any response by the authors. How does T183A and T183E behave in their settings? It would have been really nice to have this studied as well, but to help the authors to focus on other key experiments I do not consider this an essential point.
(iii) Study the phosphorylation of LATS1/2: Not addressed without any response by the authors. A change in YAP/TAZ phosphorylation should be mirrored by a change in LATS1/2 activity. I

strongly suggest that the authors at least check the phosphorylation status of LATS1 in their settings. The anti-Thr1079-P antibody from Cell Signaling works very nicely.

9) Provide substantial extensions of Figure 7.
(i) Study proliferation of knockout cells shown in Figure 3A. Does this match Fig. 7A?: Sufficiently addressed.
(ii) Study YAP knockdown in Figure 7A: Sufficiently addressed.
(iii) Does the expression of Hippo-insensitive YAP-5SA rescue the effect of siMARK4 in Figure 7A?: Sufficiently addressed.
(iv) Confirm that the effects shown in 7A and 7C are Hippo core kinase dependent. Does MST1/2 and/or LATS1/2 overexpression mimic YAP/TAZ depletion?: Not addressed without any clear response by the authors. However, given that I find the points raised above more important, I drop this point in order to help the authors to focus on the final push for this manuscript.

10) Redundancy with refs. 46, 47, and 48?: Reasonably addressed by the authors, although I still believe that some extra experiments are needed to strengthen the main conclusions of this manuscript.

Minor points: All minor points have been addressed sufficiently. Regarding point D, I suggest to keep the data presentation as it is in the revised manuscript.

2nd Revision - authors' response 27 November 2016

Response to Outstanding Referees' Comments

Referee #1: No additional comments were raised.

Referee #2: The referee requests we include the results of our overexpression screen in the manuscript as the information could be valuable to the scientific community. We believe that for the data to really be useful requires a more extensive analysis of the screen results (such as determining false positive and false negative rates, statistical analysis to indicate probability of significance of individual data points and ideally some additional validation of hits). We have not yet done this and in any case, we feel strongly that this is not pertinent to the current study. We did not present, discuss or analyze any hits other than to point out the MARKs. We have retained the screen data plot as suggested by referee #3.

Referee #3: All of the outstanding concerns raised by the referee have been addressed experimentally in the revised version as follows:

4i) The referee asked, that in addition to YAP Ser127 phosphorylation, we also examine YAP phosphorylation on Ser397/381. As requested, we used the specified antibody and now show that consistent with our results using the phospho-specific Ser127 antibody, loss of MARK4 induces an increase in YAP phosphorylation on Ser397/381 (Fig 2G). In parallel, we also assessed LATS phosphorylation on Thr1079, which was requested by the referee in point 8iii. We did not observe any changes in LATS phosphorylation upon MARK4 knockdown but speculate that the phosphorylation levels of endogenous LATS were not sufficiently high to be detected in this assay.

4iii and 5iii) As requested by the referee, we evaluated the subcellular localization of YAP/TAZ upon MARK4 depletion by biochemical fractionation. Consistent with our immunofluoresence data, abrogation of MARK4 expression either by siRNA-mediated knockdown (Fig EV2A) or using MARK4 CRISPR knockout cells (Fig EV2D), results in a dramatic loss of nuclear YAP/TAZ and an increase in cytoplasmic localization. The ratio of cytoplasmic to nuclear YAP/TAZ was quantitated and is also provided (Fig EV2A and D).

5i) As requested, we evaluated YAP phosphorylation in CRISPR knockout cells. For this we used both PhosTag gels and anti-phospho YAP (Ser127) specific antibodies. As shown in Fig 3B, MARK4 knockout cells show an increase in YAP phosphorylation as compared to Cas9 control cells. The quantitation of the blots has been included in the Extended View Data (Fig EV2B).

6ii) In response to the referee's comment regarding the presence of T-loop phosphorylation in the kinase-dead MST2; we agree with the referee that this could be attributed to phosphorylation by the endogenous MST in intact cells. Nevertheless, the in vitro kinase assay is performed after immunoprecipitation, and as is evident in Fig EV4C only the wild type and not kinase-dead MST shows an increase in T-loop phosphorylation upon addition of ATP, consistent with the idea that the assay is measuring MST autophosphorylation. Addition of purified MARK4 does not alter T-loop phosphorylation by wild type MST2, consistent with our conclusion that MARK4 does not alter MST kinase activity. This conclusion is further supported by our analysis of MST-mediated MOB1 phosphorylation as elaborated in the next comment, below.

6iv) To further investigate the role of MARK4 in regulating MST kinase activity and as suggested by the referee, we also evaluated MOB1 phosphorylation on Thr35 using the suggested specific antibody. As is shown in Fig EV4D, expression of MST2 markedly increases MOB1 phosphorylation and this increase is not changed in the presence of MARK4. This is consistent with our data showing MST autophosphorylation in the in vitro kinase assay and MST-induced SAV phosphorylation are not changed by MARK4 (Fig 6C and EV 4C). Altogether these results provide strong evidence that MST kinase activity is not affected by MARK4-induced phosphorylation.

The labelling mistake in Figure 8F has been corrected.

3rd Editorial Decision — 05 December 2016

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the report from the last critical referee that was asked to re-evaluate your study that you will find enclosed below. As you will see, also referee #3 now supports the publication of your manuscript in EMBO reports. However, the referee has one suggestion for a change in the manuscript text that we ask you to consider. Further, before we can proceed with official acceptance, there are some remaining editorial requests to be addressed.

As most of the Western panels are cut and show only a fraction of the original gel, we would prefer to have source data files for these gels. Source data is published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. Please prepare source data files (containing scans of entire gels or blots) of your experiments including size markers. Please label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

There are still thin lines present in some of the panels of Fig 2A. Can these be removed?

Although you have added a paragraph to the methods section detailing statistics, testing is only applied to some panels in Fig. 7. Also the number "n" for how many experiments were performed is not present in most of the legends. Please provide these and appropriate statistical testing for all the remaining panels (where applicable).

Some panels still miss scale bars (e.g. 7I and EV2C).

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.

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 #3:

The authors have responded experimentally to all my remaining concerns, which has significantly improved the message of this manuscript.

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Although I do not recommend further revisions, I still urge the authors to very clearly state somewhere in the manuscript (results or discussion section) that:

"they did not observe changes in Lats phosphorylation upon MARK4 deficiency, although YAP/TAZ phosphorylation and total levels were clearly altered, suggesting that Lats kinases are possibly not involved in the regulation of YAP/TAZ upon MARK4 depletion/knockout." (I think that this message is quite important, since in response to my point 8iii the authors included evidence showing that T1079 phosphorylation (which monitors Lats1 and Lats2 !) is not changed upon MARK4 removal).

3rd Revision - authors' response 22 December 2016

The authors made the requested editorial changes and resubmitted their files.

4th Editorial Decision 06 January 2017

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?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

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

B- Statistics and general methods

Question	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?	Sample size was chosen based on what is accepted and followed by investigators in the filed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
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.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
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4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	YES, number of experiments, biological replicates and pertinent statistical tests are described in figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	N/A
Is there an estimate of variation within each group of data?	YES, variations were calculated using standard deviation or standard error of mean.
Is the variance similar between the groups that are being statistically compared?	YES

C- Reagents

Question	Answer
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 this information are provided in materials 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.	Absence of mycoplasma contamination was confirmed using MycoAlret kit from LONZA. MDA-MB-231 cells were verified by STR within the last year.

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

D- Animal Models

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

E- Human Subjects

Question	Answer
11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A

14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
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).	N/A
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).	N/A
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	N/A
22. 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.	N/A

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.	N/A