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cAMP/PKA signaling reinforces the LATS-YAP pathway to fully suppress YAP in response to actin cytoskeletal changes

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Editor: David del Alamo

1st Editorial Decision 07 December 2012

Thank you for your patience while your manuscript has been reviewed. We have just now received the full set of reports from the referees, which I copy below.

As you can see from their comments, while referee #1 is rather negative towards your study, referees #2 and #3 are very supportive of its publication and agree on the high potential interest of your findings. In general, although they believe that the evidence presented properly supports your conclusions, some technical concerns have arisen with which you will have to deal before your manuscript is ready for publication. As referee reports are quite explicit I will not repeat their arguments here, but I would like to draw your attention to points 1 and 6 from referee #2 -regarding Lats phosphorylation site and the role of NF2 respectively- and major point 1 from referee #3 -on the effects of PKA on Lats activity- as important concerns that need to be adequately addressed.

Given the referees' comments and recommendations, I would like to invite you to submit a revised version of the manuscript. Please be aware that your revised manuscript must address the referees' concerns and their suggestions should be taken on board. It is 'The EMBO Journal' policy to allow a single round of revision only and, therefore, acceptance or rejection of your study will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you again for the opportunity to consider your work for publication. I look forward to your revision.

Please, do no hesitate to contact me in case you have any further question, need further input or any problem arises during the revision process.

REFEREE REPORTS

Referee #1

This MS is a confusing combination of different things.
First: finding that loss of LATS decreases YAP phosphorylation is largely expected. The increase of YAP phosphorylation by Latrunculin or Detachment has been already published. Despite the elegance of the experimental set-up in Figure1, this does not advance the field forward. Moreover, the critical missing piece of evidence is the complete lack of functional characterization of the new allele here presented.
Second, in keeping with the lack of causal relationships, it is very likely, for example, that phosphorylation may result from increased residency of YAP in the cytoplasm. The data does not tell us anything about the mechanism by which Latrunculin or Detachment regulate YAP.
Third: what is the phenotype of the mice? This is quite important.
Fourth: the whole PKA connection is very confusing. The fact that forskolin treatment induces YAP-Phosph. is already known; Again less clear is to what extent all this is direct and relevant. There are tons of biological consequences downstream of pharmacological modulation of a kinase like PKA! Moreover, looking at Figure 2D and Figure 4C, I cannot see how one can get convinced that anything substantial is really going on, let alone the fact that this is through YAP (as opposed to an integration of parallel events).
Fifth: Figure 5 is missing essential controls. Again, the functional relevance of AMOTs is unknown, as well as its eventual connection/dependency on YAP.
Also unknown is how all the multiple pieces are making sense collectively: detachment, cytoskeleton, NF2, AMOT, PKA. This is so spread-out.

Referee #2

This is a very interesting manuscript that describes a novel role for PKA signaling in the LATS-YAP pathway. A major mechanism for regulation of YAP is through the Hippo pathway and the authors show here that PKA plays a major role in regulating LATS which in turn phosphorylates YAP and inhibits its ability to serve as a transcriptional activator. Because YAP is a potent oncogene that drives cell proliferation, these studies have considerable significance and introduce new levels of mechanistic understanding. The authors focus on two phosphorylation sites in YAP that are important for inhibition of YAP activity. One is Ser127 which was shown previously to be a 14-3-3 binding site and would thus promote cytoplasmic retention. The other site is Ser381 and this is the focus of the present paper. This site is phosphorylated by LATS and it is PKA that regulates LATS and leads to its activation and YAP inhibition. The authors show initially that cytoskeleton damage leads to the inactivation of YAP and that the complex with Mob1 is essential; however, upstream components of the Hippo pathway are dispensable, including NF2. They then go on to show that PKA is specifically responsible for the activation of LATS and that the target for LATS following PKA activation is Ser381 on YAP. In addition to H89, which inhibits PKA but is fairly non-specific, they use a dominant negative form of the PKA RIa subunit to confirm that PKA is the upstream activating kinase for LATS. To show that active PKA can suppress phenotypes associated with YAP over-expression, in particular the induction of stem cell neural progenitors, they also use a mutant form of the PKA C subunit that is weakly inhibited by the regulatory subunits. They then ask

whether NF2, which was reported previously to be an AKAP, is required for the PKA-mediated inhibition of YAP. To do this they transfect 293 cells with wt NF2 and with an NF2 mutant that can no longer bind to PKA. This mutant failed to induce Ser381 phosphorylation. The results are compelling and define a new PKA-mediated mechanism for regulation of YAP. Although the results are mostly convincing, there are some fundamental points that need to be addressed before publication can be recommended.

Reviewer concerns.

1. While the authors show that PKA is likely the activator of LATS, they do not ever address the question of where the PKA phosphorylation site is located. They indicated with antibodies that it is not the activation loop nor is it the HF motif. They need to indicate where the likely site is. Is there a putative PKA site and does mutation of this site abolish the phosphorylation of Ser381? What about the turn motif? Is this phosphorylated in LATS? There needs to be a much more comprehensive discussion of the phosphorylation sites in LATS which is the major target for PKA. How does the PKA phosphorylation of LATS prime it for then phosphorylating YAP? Does it create a binding site for YAP? What other kinases are involved in the activation of LATS?

2. The authors show that phosphorylation of the Activation Loop is not effected by H89 but at the same time they show that phosphorylation of the HF motif is dramatically increased in the presence of H89. They comment on this as being surprising, but they must further explain this. What phosphorylates the HF motif and why is this process inhibited by PKA inhibitors? It seems as though this could be an important part of the mechanism. It cannot be glossed over. What kinase phosphorylates the AL site and what phosphorylates the turn motif? These events tend to be highly regulated.

3. The authors also do not discuss the possible functional consequences of the Ser381phosphorylation site. Are there any clues as to why this site is so important?

4. Figure 3. What do the S and L indicate? Soluble and whole cell lysate fractions?

5. Although H89 does inhibit PKA, it is a remarkably non-specific inhibitor. The authors should use the PKI inhibitor peptide, which can be induced or the myristylated form, which can be taken up by cells. This is a far superior way of demonstrating PKA specificity.

6. The authors show in the beginning that the knockout of NF2 does not effect phosphorylation of Ser381, and yet they show later on in 293 cells that NF2 does not facilitate the phosphorylation of Ser381 when the binding site for PKA is deleted. They also indicate in the text that the binding is preferential for RII even though the earlier report of NF2 being an AKAP said that it was specific for RI. Actually Figure S10 does not show a preference for the R2 subunits although the results with RIa are not indicated. This is contrary to what they say in the text. Both of these inconsistencies need to be explained. If NF2 is an essential AKAP for bringing PKA to the complex, then why did the depletion of NF2 not make a difference? Is there another AKAP? What endogenous R-subunits do they pull down with the LATS-YAP complex? They should also use the AKAP disrupting peptides for this experiment as these should work for the endogenous proteins as well. There are disrupting peptides that can also discriminate between RI and RII specific AKAPs.

7. It is also essential that the authors reference the Zhang et al paper and point out specifically how their results build on that paper. Omission of this important reference is a serious concern. (Zhang, H, et al (2012) Developmental Biology 361:103-115).

Referee #3

Kim et al show an involvement of PKA as an upstream activator/amplifier of Lats1/2. They start by showing that the disruption of F-actin leads to a dramatic change in serine 381 phosphorylation of YAP. Through the use of Lats1/2 knockout cells, they show that PKA affects serine 381 phosphorylation through Lats1/2. They go on to show that in processes that depend on Yap regulation (anoikis, serum starvation and progenitor cell formation in the neural tube), PKA activity counteracts YAP activity. Finally, they show that PKA is not only mediating the effects of actin and

FBS on YAP but also of other Hippo pathway regulators like NF2 and angiomotin family proteins.

The study is interesting and overall I think that the experiments are well controlled and that the author's conclusions are mostly valid. However, I do have some concerns that I would like to have addressed before this manuscript warrants publication.

Major concerns:
1) A direct test of the hypothesis that PKA acts on Yap by modulating the activity of Lats1/2 is presented in figure 3D. However, I have questions about the results and the interpretation of the data. Figure 1A and figures 2A-C show a switch-like change in serine 381 phosphorylation that is mediated by PKA but a modest change in serine 127 phosphorylation that seems PKA independent. However, figure 3D shows that PKA modulates the kinase activity of Lats1/2 both for their activity towards serine 127 and towards serine 381. The authors need to explain this difference. In other words: does PKA activate the kinase activity of Lats1/2 in general or does it mainly facilitate the Lats1/2 mediated phosphorylation of serine 381 but not serine 127?

2) The authors also need to exclude the possibility that the phosphorylation of serine 127 and 381 in figure 3D is due to undetected PKA that may still be left in the reaction. In support of the authors' conclusions that PKA acts through Lats1/2, figure 2F indicates that PKA is unable to phosphorylate Yap in Lats1/2 knockout cell lines. To exclude however that PKA can directly phosphorylate Yap, the authors need to show that PKA is still active in the experiment in figure 2F, which would indicate that although PKA is active, it is unable to phosphorylate YAP.

Other concerns:
1) A general concern is that quite a few experiments are poorly explained. For example, it is not clear what S and L notations are next to blots, nor that p-PKA sub is an antibody that recognizes all phosphorylated PKA consensus sites, but that in most of the experiments here, it relates to the phosphorylation of Lats specifically. The authors need to clearly explain lanes 5 in figure 3a, figure 3D.

2) Figure 1B: There is still YAP phosphorylation in Lats knockout cells. Please explain. In contrast, no YAP phosphorylation is detected in figure 2F. Please clarify the difference between these experiments.

3) Indicate whether the experiments in figure 1 are performed with confluent cells or not. Do you see conditions where S127 is not phosphorylated?

4) Figure 1D: the loading control looks very variable and correlates with the phosphorylation status of S381. Explain.

5) Figure 2D: the pictures are low resolution and very dark. It is difficult for the reader to draw conclusions from these pictures.

6) Figure 4A: label to make it clear we are looking at Yap S127A.

7) Figure 4E: removal of FBS stimulation is not called anoikis. Please modify the text to say that PKA modulates the response to anoikis and serum starvation.

8) Figure 4F-G: It appears that Sox2 is also induced in cells that are not transfected. Does Yap act non cell-autonomously?

9) Figure 5A: please add a control in which no upstream component is overexpressed for reference.

10) Figure S5: explain the observation that H-89 leads to phosphorylation of LATS pT1079.

1st Revision - authors' response 06 March 2013

Referee #1

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Third: what is the phenotype of the mice? This is quite important.
Fourth: the whole PKA connection is very confusing. The fact that forskolin treatment induces YAP-Phosph. is already known; Again less clear is to what extent all this is direct and relevant. There are tons of biological consequences downstream of pharmacological modulation of a kinase like PKA! Moreover, looking at Figure 2D and Figure 4C, I cannot see how one can get convinced that anything substantial is really going on, let alone the fact that this is through YAP (as opposed to an integration of parallel events).

Responses concerning the comments written above:

1) As the referee have mentioned, the conclusions drawn from figure 1 is largely expectable. However, it should be noted that previous reports have conflicts on the requirement of LATS1/2 in the context of actin cytoskeletal disruption; Piccolo's group suggested that LATS1/2 are dispensable (Dupont et al, 2011) whereas Guan's group concluded that they are required (Zhao et al, 2012). This conflict is mainly due to the use of siRNA approach rather than genetic knock-out. In figure 1, using genetically deficient cell lines, which is a superior experimental system than siRNA, we provide definite proof that LATS1/2-Mob1 complex is indeed essential.

2) Even though previous studies reported that actin cytoskeletal damages such as latrunculin B, cytochalasin D, or maintenance of cells in suspension, inactivates YAP, signaling events downstream of the actin cytoskeleton are poorly understood. This study newly revealed the novel role of PKA in regulating LATS-YAP pathway. Of course modulation of PKA can result in diverse effects and therefore direct relationship between PKA and YAP should be thoroughly proven. In our original MS, we provided evidences to show that PKA directly phosphorylates LATS both *in vitro* and *in vivo* and this potentiates LATS kinase activity (Figure 3). Furthermore, in our revised MS, we have defined PKA target residues on LATS2. The mutant LATS2 was not phosphorylated by PKA both *in vitro* and *in vivo* but retained other regulations. Importantly, this mutant failed to effectively induce YAP Ser381 phosphorylation by actin damages when reconstituted in Lats1/2 deficient MEFs and showed weaker kinase activity *in vitro* compared to LATS2 WT (Related figures are provided in Figure 4 in our revised MS. More detailed demonstration on this issue is also provided as response to comment 1 by referee #2). Taken together, we provide sufficient and compelling evidences that PKA directly acts on LATS. All these findings provide novel mechanistic insight into how latrucunlin B or cell detachment regulates YAP.

3) Organismal phenotype is very important point, but it does not necessarily have to be mice. Although we did not have chance to dissect this in mouse model, we instead utilized the chick embryo system. As illustrated in figure 5F-5I (In revised MS), YAP and PKA functionally cooperated to regulate neural stem/progenitor pool in developing chick embryos. Therefore, we provide compelling evidence that YAP and PKA functionally cooperate in animal model. Analysis of the phenotypes of LATS1/2 dKO mice might go beyond the scope of this study.

4) Figure 2D clearly shows that the localization of YAP becomes dispersed by PKA agonist. This conclusion is also supported by the NC fractionation data in figure 2C. The resolution of image was also pointed out by referee 3 and we replaced the image with higher resolution.

5) We examined the functional cooperation between YAP and dnPKA using various assays which are illustrated in figure 5 (Revised MS). Although the effect is somewhat marginal in panel C, we

nevertheless obtained reproducible results in independent trials which reached statistical significance. In addition, one can see obvious effects in other panels of figure 5. Of course, the extent of effect can vary depending on the nature of specific assay. However, the important point is that it is reproducible, statistically significant, and confirmed by various assays.

Fifth: Figure 5 is missing essential controls. Again, the functional relevance of AMOTs is unknown, as well as its eventual connection/dependency on YAP.

Response: This comment was also raised by reviewer #3. In original MS, we provided the control experiments in supplementary figure, because they are repeated for NF2, AmotL1, and AmotL2. As suggested, we now present new figure including those control lanes.

Also unknown is how all the multiple pieces are making sense collectively: detachment, cytoskeleton, NF2, AMOT, PKA. This is so spread-out.

Response: I respectfully disagree with this comment. From figure 1 to figure 4, we clearly demonstrated that PKA is involved in actin cytoskeleton damage-induced signaling by activating LATS. In figure 5, we showed that PKA and YAP functionally cooperate. Finally in figure 6, we tested whether PKA activity is involved in LATS-YAP pathway in general. NF2 and AmotL1/2 were used here to activate the canonical Hippo pathway. In all combinations, blocking PKA activity abolished YAP phosphorylation induced by these upstream factors. Thus, we believe our study is clearly organized.

Referee #2

This is a very interesting manuscript that describes a novel role for PKA signaling in the LATS-YAP pathway. A major mechanism for regulation of YAP is through the Hippo pathway and the authors show here that PKA plays a major role in regulating LATS which in turn phosphorylates YAP and inhibits its ability to serve as a transcriptional activator. Because YAP is a potent oncogene that drives cell proliferation, these studies have considerable significance and introduce new levels of mechanistic understanding. The authors focus on two phosphorylation sites in YAP that are important for inhibition of YAP activity. One is Ser127 which was shown previously to be a 14-3-3 binding site and would thus promote cytoplasmic retention. The other site is Ser381 and this is the focus of the present paper. This site is phosphorylated by LATS and it is PKA that regulates LATS and leads to its activation and YAP inhibition. The authors show initially that cytoskeleton damage leads to the
inactivation of YAP and that the complex with Mob1 is essential; however, upstream components of the Hippo pathway are dispensable, including NF2. They then go on to show that PKA is specifically responsible for the activation of LATS and that the target for LATS following PKA activation is Ser381 on YAP. In addition to H89, which inhibits PKA but is fairly non-specific, they use a dominant negative form of the PKA RIa subunit to confirm that PKA is the upstream activating kinase for LATS. To show that active PKA can suppress phenotypes associated with YAP over-expression, in particular the induction of stem cell neural progenitors, they also use a mutant form of the PKA C subunit that is weakly inhibited by the regulatory subunits. They then ask whether NF2, which was reported previously to be an AKAP, is required for the PKA-mediated inhibition of YAP. To do this they transfect 293 cells with wt NF2 and with an NF2 mutant that can no longer bind to PKA. This mutant failed to induce Ser381 phosphorylation. The results are compelling and define a new PKA-mediated mechanism for regulation of YAP. Although the results are mostly convincing, there are some fundamental points that need to be addressed before publication can be recommended.

Reviewer concerns.

1. While the authors show that PKA is likely the activator of LATS, they do not ever address the question of where the PKA phosphorylation site is located. They indicated with antibodies that it is not the activation loop nor is it the HF motif. They need to indicate where the likely site is. Is there a putative PKA site and does mutation of this site abolish the phosphorylation of Ser381? What about the turn motif? Is this phosphorylated in LATS? There needs to be a much more comprehensive discussion of the phosphorylation sites in LATS which is the major target for PKA. How does the

PKA phosphorylation of LATS prime it for then phosphorylating YAP? Does it create a binding site for YAP? What other kinases are involved in the activation of LATS?

Response: This is very excellent comment. Indeed, the phosphorylation site issue was the most critical shortcoming in our original MS. To address this issue, we newly identified the target sites at which LATS is phosphorylated by PKA and showed its contribution to LATS-YAP signaling. We added new figure in the revised MS which describes our new findings concerning the phosphorylation site. (All the figure numbers described below refer to the revised MS).
PKA optimally phosphorylates RRxS/T motifs and in some cases Lysine can substitute for Arginine. In LATS2, there exist two RRxS/T motifs (S172 and S380) and two weak consensus motifs (S584 and S592), all of which locate N-terminal to the kinase domain. We substituted all four Serines to Alanines to generate LATS2 4SA mutant. *In vitro*, LATS2 4SA mutant was not phosphorylated by purified PKA (Figure 4A). More importantly, LATS2 4SA was not phosphorylated by PKA in response to cell detachment (Figure 4B). In contrast, LATS2 4SA was normally phosphorylated at both activation loop and hydrophobic motif by cell detachment. LATS2 4SA also normally interacted with Mob1 (Figure 4B and Supplementary Figure S8). Thus, LATS2 4SA is specifically defective in phosphorylation by PKA and retains other regulatory mechanisms. To ask the impact of the mutation in cytoskeletal damage mediated signaling, we reconstituted immortalized Lats1/2-null MEFs with either LATS2 WT or LATS2 4SA constructs. LATS2 4SA reconstituted cells showed attenuated YAP phosphorylation in response to latrunculin B or cell detachment (Figure 4C). Notably, in this setting where endogenous Lats1/2 are deleted and only LATS2 4SA is present, both Ser127 and Ser381 phosphorylations were attenuated. However, the effect was much more pronounced for Ser381 phosphorylation. Lastly, we compared kinase activity between LATS2 WT and LATS2 4SA using immune-complex prepared from 293T cell and found that the enzymatic activity of LATS2 4SA is remarkably lower (Figure 4D). In conclusion, we have mapped the PKA target sites on LATS2 and proved that this phosphorylation is required for efficient YAP phosphorylation in response to actin cytoskeletal damage.

Yet, we do not know how PKA mediated phosphorylation enhances LATS activity. We notice that although PKA enhanced LATS2 activity *in vitro* (Figure 3D), the effect was quite weaker than what we observed in intact cells by PKA inhibitors. This suggests missing or limiting components in the *in vitro* assay. Therefore, we speculate that phosphorylation on LATS by PKA mediates interaction with yet unidentified complex component(s). Mass spectrometry analysis using LATS2 WT or LATS2 4SA as bait might identify such component(s). In the revised MS, we explained this point in the discussion section.

2. The authors show that phosphorylation of the Activation Loop is not effected by H89 but at the same time they show that phosphorylation of the HF motif is dramatically increased in the presence of H89. They comment on this as being surprising, but they must further explain this. What phosphorylates the HF motif and why is this process inhibited by PKA inhibitors? It seems as though this could be an important part of the mechanism. It cannot be glossed over. What kinase phosphorylates the AL site and what phosphorylates the turn motif? These events tend to be highly regulated.

Response: These are very important and central questions in the field. Activation loop of LATS is auto-phosphorylated and Mob1 binding is thought to stimulate this reaction. For the hydrophobic motif, MST1/2 can phosphorylate the HM site in the context of canonical Hippo pathway. However, MST1/2 does not seem to be the sole kinase that phosphorylates the hydrophobic motif. As described in our paper, Mst1/2 deficient MEFs still normally phosphorylated YAP. One unpublished data we have is that when LATS1 T1079A mutant (hydrophobic motif mutant) was complemented to Lats1/2-null MEFs, it still could not rescue YAP phosphorylation. These results indicate that there exists another HM kinase. In agreement with our observations, Avruch's group also has reported Mst1/2 independent HM phosphorylation by cell density (Zhou et al, 2009). Thus, it is likely that H-89 increases the HM phosphorylation in LATS by activating LATS HM kinase. Identification of the LATS HM kinase is one of the most important questions in the field and we are currently trying to identify the kinase. However, we believe that this might go beyond the scope of the current paper.

3. The authors also do not discuss the possible functional consequences of the Ser381phosphorylation site. Are there any clues as to why this site is so important?

Response: The importance of Ser381 phosphorylation has been demonstrated by Zhang et al. Ser381 phosphorylation mediates YAP degradation by ubiquitin pathway (Zhao et al, 2010). Mutagenesis study has shown that mutating all five HxRxxS/T motifs in YAP confers transforming activity. However, if either Ser127 or Ser381 are intact (while other four motifs are still mutated), it fully abrogates the transforming activity of YAP 5SA (Zhao et al, 2009). Therefore, Ser381 phosphorylation as well as Ser127 phosphorylation is very important to suppress YAP activity. In our study, we showed that inhibiting PKA activity decreased YAP Ser381 phosphorylation. Therefore, we tried to see if YAP is stabilized in PKA inhibited cells by cytoskeletal damaging agents. We tested this by looking at total YAP protein level in cells incubated with Cycloheximide plus Latrunculin B with or without H-89 pre-treatment. We did observe some tendency, but the effect was very subtle. Over the study, PKA seems to play supportive role in augmenting LATS activity. Although YAP phosphorylation kinetics is attenuated in PKA inhibited cells (or cells expressing PKA non-phosphorylatable LATS2), significant phosphorylation of YAP ultimately occurs. Therefore, at 2 hour or 6 hour time points after cytoskeletal damage at which we examined YAP protein level, only marginal effects are observed.

4. Figure 3. What do the S and L indicate? Soluble and whole cell lysate fractions?

Response: We apologize for the omission. S stands for 'Short exposure image' and L stands for 'Long exposure image'. We changed the labeling to SE and LE respectively and modified the figure legends to indicate what SE and LE stands for.

5. Although H89 does inhibit PKA, it is a remarkably non-specific inhibitor. The authors should use the PKI inhibitor peptide, which can be induced or the myristylated form, which can be taken up by cells. This is a far superior way of demonstrating PKA specificity.

Response: We agree with the referee's comment that PKI is a reliable way to specifically inhibit PKA. We were skeptical about using lipid-linked peptides such as myristoylation or stearylation. Although these modifications can allow the peptides to enter the cell, the peptides are likely to anchor in the membrane and not to diffuse inside the cell. Therefore, one might observe PKA inhibition effect only when the signaling event is activated very close to the plasma membrane. In fact, when we pre-treated cells with Myr-PKI followed by latrunculin B treatment, we could not see any inhibition of YAP phosphorylation. In addition, Myr-PKI pre-treatment could not inhibit Forskolin/IBMX induced YAP phosphorylation as well. This suggests that PKA-LATS signaling is activated in subcellular compartment where Myr-PKI cannot efficiently reach.
Therefore, we thought that poly-Arg linkage would be superior way of inhibiting PKA activity in the cell. This strategy was previously used in other studies too (Lu et al, 2007; Matsushita et al, 2001). To deliver PKI into cells, we synthesized PKI peptide with 11 Arignines (11R-PKI) to the N terminus. Cells can uptake polybasic peptides via membrane channels (This strategy is also used for AKAP inhibitor peptides, which is the next comment). In supplementary figure S3, we show that pre-treatment of 11R-PKI attenuated YAP Ser381 phosphorylation albeit weaker than H-89. We could not test the effect of 11R-PKI in detached cells because trypsin treatment will digest the peptide (We added H-89 during the trypsinization step in figure 2A). In addition, we modified figure 6A (in the revised MS) to include 11R-PKI next to H-89. 11R-PKI treatment in 293T cells transfected with YAP, LATS1 and Hippo upstream factors also reduced YAP Ser381 phosphorylation albeit weaker than H-89. In figure 6C, we also used RSV-PKI plasmid to show that inducing PKI inside the cell abolished YAP phosphorylation. Expression of PKI was confirmed by immunoblotting the whole transferred membrane against phospho-PKA substrate antibody, which showed overall reduction in PKI transfected cell. Collectively, using both induced and delivered PKI, we show that PKI can attenuate YAP phosphorylation induced by cytoskeletal damage or Hippo pathway activation.

6. The authors show in the beginning that the knockout of NF2 does not effect phosphorylation of Ser381, and yet they show later on in 293 cells that NF2 does not facilitate the phosphorylation of Ser381 when the binding site for PKA is deleted. They also indicate in the text that the binding is preferential for RII even though the earlier report of NF2 being an AKAP said that it was specific for RI. Actually Figure S10 does not show a preference for the R2 subunits although the results with RIa are not indicated. This is contrary to what they say in the text. Both of these inconsistencies need to be explained. If NF2 is an essential AKAP for bringing PKA to the complex, then why did

the depletion of NF2 not make a difference? Is there another AKAP? What endogenous R-subunits do they pull down with the LATS-YAP complex? They should also use the AKAP disrupting peptides for this experiment as these should work for the endogenous proteins as well. There are disrupting peptides that can also discriminate between RI and RII specific AKAPs.

Response: These are wonderful suggestions. In the original MS, we showed that over-expressed Regulatory Subunits and LATS associate in intact cells, thereby implicating AKAP in LATS regulation. Next, we showed that NF2 can function as such AKAP since deletion of its AKAP domain abolished YAP Ser381 phosphorylation although we did not rule out existence of other AKAPs. The core question is to define by which type of AKAP LATS is regulated. Two experimental approaches were suggested; investigating protein interactions in endogenous level and using AKAP inhibitor that can discriminate specific AKAPs.

First, we originally re-examined the Gronholm's study but did not include the result. In our hands, NF2 also efficiently associated with Type 2 Regulatory Subunits as well as Type 1. We now present this result in supplementary figure S14.

Second, NF2 depletion had no impact on YAP phosphorylation induced by latrunculin B although its AKAP domain was required for YAP phosphorylation in over-expression study. One possibility we have described in the discussion section is that other FERM domain proteins might function redundantly with NF2. Many FERM domains commonly possess AKAP domains (Dransfield et al, 1997; Neisch & Fehon, 2011). In drosophila, NF2 functions redundantly with another FERM domain protein Expanded which is also conserved in the mammals (Angus et al, 2012; Hamaratoglu et al, 2006). However, at the same time, we still open the possibility that other protein might function as AKAP to facilitate PKA-LATS-YAP signaling in response to actin damage. It will be important to identify exact AKAP(s) in the future study.

Third, we extensively performed co-IP studies with endogenous proteins. Yet, we could not detect endogenous interaction between LATS1/2 and Type 2 subunits (Type 1 subunits were unexamined due to absence of antibody). However, we have never detected endogenous interaction between NF2 and LATS1/2 either. Many previous studies suggest that NF2 and LATS should function within a complex and we do detect their interaction in over-expression systems. Therefore, it is likely that the AKAP-PKA-LATS complex is vulnerable and weakly interacts in the lysis condition.

Lastly, we synthesized Arg11 linked peptides that can disrupt either type 1 or type 2 AKAPs specifically, named RIAD-11R and 11R-SuperAKAP-IS respectively. These peptides were previously designed by massive iterative peptide screening by John D. Scott (Carlson et al, 2006; Gold et al, 2006). Pre-treating either RIAD-11R or 11R-SuperAKAP-IS alone did not impair YAP phosphorylation induced by latrunculin B. However, when the peptides were pre-tread in combination, YAP Ser381 phosphorylation was markedly attenuated (Supplementary Figures S13B). This suggests that the responsible AKAP should be dual specificity AKAP. We further validated our conclusion by showing that pre-treatment of RIAD-11R and 11R-SuperAKAP-IS abolished phospho-PKA substrate signal in LATS2 pull-down sample (Supplementary Figure S13C).

In addition, we reorganized the manuscript to describe the possible involvement of AKAPs more clearly to the readers. We moved original supplementary figure S2B to supplementary figure S15. We extensively discussed about the how and which AKAPs are likely to function in the PKA-LATS-YAP pathway.

7. It is also essential that the authors reference the Zhang et al paper and point out specifically how their results build on that paper. Omission of this important reference is a serious concern. (Zhang, H, et al (2012) Developmental Biology 361:103-115).

Response: We apologize for missing this paper in original MS. In general, Zhang's paper extended the previous findings in chick neural tube to mice/mammalian neural system; importance of YAP inhibition for cell cycle exit of neural progenitors and differentiation. The YAP-PKA connection might be especially important in neuronal system because many studies show that PKA activity promotes neuronal cell differentiation in various systems (Cox et al, 2000; Kim et al, 2005; Li et al, 2007; Suh et al, 2001; Vaudry et al, 2002). Therefore, it is very likely that neuronal-differentiation inducing PKA agonists, such as PACAP, acts at least in part by inhibiting YAP. How the proneural gene activities (Ascl1 or Neurogenin2) impinge on LATS1/2 are unidentified in Zhang's paper, however PKA should be involved since proneural genes are destabilizing YAP. Also it is interesting to note that only LATS activation but not that of MST1/2 was prominent during neuron differentiation. Upstream of LATS1/2 might differ from the canonical Hippo pathway in neuronal

system. In this revised MS, we newly add these points in our discussion section with proper references. We also referenced Zhang's paper in the results section while explaining the chick data and while mentioning the cell non-autonomous effect of YAP as this phenomenon was also noticed by Zhang's study (Also refer to comment #8 of referee #3).

Referee #3

Kim et al show an involvement of PKA as an upstream activator/amplifier of Lats1/2. They start by showing that the disruption of F-actin leads to a dramatic change in serine 381 phosphorylation of YAP. Through the use of Lats1/2 knockout cells, they show that PKA affects serine 381 phosphorylation through Lats1/2. They go on to show that in processes that depend on Yap regulation (anoikis, serum starvation and progenitor cell formation in the neural tube), PKA activity counteracts YAP activity. Finally, they show that PKA is not only mediating the effects of actin and FBS on YAP but also of other Hippo pathway regulators like NF2 and angiomotin family proteins. The study is interesting and overall I think that the experiments are well controlled and that the author's conclusions are mostly valid. However, I do have some concerns that I would like to have addressed before this manuscript warrants publication.

Major concerns:
1) A direct test of the hypothesis that PKA acts on Yap by modulating the activity of Lats1/2 is presented in figure 3D. However, I have questions about the results and the interpretation of the data. Figure 1A and figures 2A-C show a switch-like change in serine 381 phosphorylation that is mediated by PKA but a modest change in serine 127 phosphorylation that seems PKA independent. However, figure 3D shows that PKA modulates the kinase activity of Lats1/2 both for their activity towards serine 127 and towards serine 381. The authors need to explain this difference. In other words: does PKA activate the kinase activity of Lats1/2 in general or does it mainly facilitate the Lats1/2 mediated phosphorylation of serine 381 but not serine 127?

Response: This is excellent question concerning our final model. At this stage, we prefer the model that PKA phosphorylation of LATS1/2 enhances its activity in general. Observations favoring this model are 1) *In vitro* kinase assay in figure 3D show that PKA phosphorylated LATS2 have increased activity toward both YAP Ser127 and Ser381, 2) LATS2 4SA complementation in Lats1/2 deficient MEFs (Issue raised by reviewer #2 as first comment) resulted in attenuation of both S127 and S381 phosphorylation, although Ser381 phosphorylation was more strongly affected. These results suggest that PKA phosphorylated LATS2 have general increase in its activity. We think that because Ser127 phosphorylation by LATS is biochemically more preferable, inhibition of PKA in LATS1/2 proficient cells resulted in selective impairment of Ser381 phosphorylation (There are sufficient LATS kinase present in the cell to induce Ser127 phosphorylation even when PKA is inhibited). However, when overall LATS quantities are significantly reduced (Figure 4C), Ser127 phosphorylation is also attenuated. We explain this point in the discussion section of the revised MS.

2) The authors also need to exclude the possibility that the phosphorylation of serine 127 and 381 in figure 3D is due to undetected PKA that may still be left in the reaction. In support of the authors' conclusions that PKA acts through Lats1/2, figure 2F indicates that PKA is unable to phosphorylate Yap in Lats1/2 knockout cell lines. To exclude however that PKA can directly phosphorylate Yap, the authors need to show that PKA is still active in the experiment in figure 2F, which would indicate that although PKA is active, it is unable to phosphorylate YAP.

Response: These are very important control experiments to fully validate our conclusion. To rule out the possibility that residual PKA in the tube is responsible for the additional phosphorylations seen in lane 2 and 7 of figure 3D, we repeated the experiment except that we included PKI (5-24) in the second kinase assay reaction buffer. PKI (5-24) is a potent peptide inhibitor of PKA. We now present this result in supplementary figure S7. Lanes 5 and 6 clearly show that PKI efficiently inhibited PKA activity. In this condition, we were able to recapitulate the result of figure 3D.
Next, to examine PKA activity in Lats1/2-null MEFs, we performed western blot against phospho-CREB (Ser133) which is a well known substrate of PKA. In figure 2F of the revised MS, we show comparable CREB phosphorylation in control and Lats1/2-null MEFs indicating that PKA itself is still active in Lats1/2 deficient cells.

Other concerns:
1) A general concern is that quite a few experiments are poorly explained. For example, it is not clear what S and L notations are next to blots, nor that p-PKA sub is an antibody that recognizes all phosphorylated PKA consensus sites, but that in most of the experiments here, it relates to the phosphorylation of Lats specifically. The authors need to clearly explain lanes 5 in figure 3a, figure 3D.

Response: We apologize for our omissions. S stands for 'Short exposure image' and L stands for 'Long exposure image'. We changed the labeling to SE and LE respectively and modified the figure legends to indicate what SE and LE stand for. In figure 3A and 3D, LATS1 or LATS2 were prepared by immunoprecipitation from transfected 293T cells. Therefore, western blotting against phospho-PKA substrate antibody detects phosphorylated LATS1/2 only. We realize that the labeling in the original MS was obscure and thank to the referee for noting our carelessness. We modified the labeling and figure legends to clarify the experimental scheme.

2) Figure 1B: There is still YAP phosphorylation in Lats knockout cells. Please explain. In contrast, no YAP phosphorylation is detected in figure 2F. Please clarify the difference between these experiments.

Response: The amount of residual YAP phosphorylation can differ between independent experiments depending on the efficiency of Lats2 deletion by Cre expression, or film exposure time. This is especially so for Ser127 phosphorylation, which is extremely sensitive to trace amount of Lats2 *in vivo*. In case of figure 1B compared to figure 2F, there's some residual Lats2 in figure 1B. In addition, the YAP phospho-Ser127 blot in Latrunculin B treated set of figure 1B is exposed longer than that of detachment set of figure 1B or figure 2F (Basal phospho-Ser127 signal is stronger in non-stimulated cells). In the revised MS, we prepared independent Lats1/2 deficient MEF and treated them with latrunculin B. We now replace latrunculin B experiment set in figure 1B to one that does not show residual YAP phosphorylation.

3) Indicate whether the experiments in figure 1 are performed with confluent cells or not. Do you see conditions where S127 is not phosphorylated?

Response: We thank to the reviewer for picking up our omission of important experimental condition. Cell confluence is indeed important issue in the field. Throughout the study, we carried out all our treatments (Latrunclin B, detachment, Forksokolin/IBMX, and GPCR agonists) in about 80% confluent cells. We now mention this condition in the first subsection of 'Materials and Methods' section.
Personally, we realized that when cells are seeded in extremely low density (about 5%), YAP Ser127 phosphorylation is quite reduced. Yet, we are not sure whether this is due to the extensive cell spreading effect in low density cultured cells or true cell-cell contact elicited effect. We observed that Ser127 phosphorylation tends to saturate as cells grow little bit denser. Thus, we used experimental condition where Ser127 phosphorylation level seemed to be saturated.

4) Figure 1D: the loading control looks very variable and correlates with the phosphorylation status of S381. Explain.

Response: We believe that the reviewer is commenting on the detachment experiment set. In that particular experiment, we obtained too little amount of cell lysates of low protein concentration. Therefore, large errors should have occurred in the process of measuring protein concentrations. However, the strict loading control to compare phospho-YAP levels is total YAP level not b-actin. In figure 1D, although b-actin levels are lower in attached cells, one can see that total YAP level is decreasing over time. Therefore, normalizing phospho-YAP by total YAP level indicates increase of YAP phosphorylation. For this reason, these data shown does not hamper our conclusion that Mst1/2 are dispensable for YAP phosphorylation induced by cell detachment. In agreement with our data, Guan's group also reported same result in their previous study (Zhao et al, 2012).

5) Figure 2D: the pictures are low resolution and very dark. It is difficult for the reader to draw conclusions from these pictures.

Response: As suggested, we now replace the figure with higher resolution image.

6) Figure 4A: label to make it clear we are looking at Yap S127A.

Response: As suggested, we changed the labeling to clarify that YAP S127A is combined with dnPKA. In addition, we also changed the labeling in panel E and supplementary figure S9 to clarify the combinations used in each experiment.

7) Figure 4E: removal of FBS stimulation is not called anoikis. Please modify the text to say that PKA modulates the response to anoikis and serum starvation.

Response: We admit that the original MS described the results in somewhat confusing way. As suggested, we modified the text to clarify that figures 5D and 5E (In revised MS) refers to serum starvation.

8) Figure 4F-G: It appears that Sox2 is also induced in cells that are not transfected. Does Yap act non cell-autonomously?

Response: This is an excellent point. Indeed, we observed strong non cell-autonomous effect in the chick embryo system. In agreement with our observation, other studies have suggested non cell-autonomous effect in mammalian cell culture system or in flies. For instance, YAP promotes non cell-autonomous transformation in MCF10A cells by up-regulating Amphiregulin (Zhang et al, 2009). Also, Yorkie induces and thereby secretes unpaired (IL6) cytokine to mediate tissue regeneration in response to intestine damage or mitochondrial malfunction (Ohsawa et al, 2012; Staley & Irvine, 2010). In the revised MS, we newly noted the non cell-autonomous effect in the chick experiment and referenced the aforementioned papers.

9) Figure 5A: please add a control in which no upstream component is overexpressed for reference.

Response: In original MS, we provided the control experiments in supplementary figure, because they are repeated for NF2, AmotL1, and AmotL2. As suggested, we now provide new figure including those control lanes. In addition, we extend the figure by adding 11R-PKI treated samples next to H-89 as response to comment #5 of referee #2.

10) Figure S5: explain the observation that H-89 leads to phosphorylation of LATS pT1079.

This comment is also mentioned by referee #2 (comment #2). Please refer to our response to comment #2 of referee #2. Activation loop of LATS is auto-phosphorylated and Mob1 binding is thought to stimulate this reaction. For the hydrophobic motif, MST1/2 can phosphorylate the HM site in the context of canonical Hippo pathway. However, MST1/2 does not seem to be the sole kinase that phosphorylates the hydrophobic motif. As described in our paper, Mst1/2 deficient MEFs still normally phosphorylated YAP. One unpublished data we have is that when LATS1 T1079A mutant (hydrophobic motif mutant) was complemented to Lats1/2-null MEFs, it still could not rescue YAP phosphorylation. These results indicate that there exists another HM kinase. In agreement with our observations, Avruch's group also has reported Mst1/2 independent HM phosphorylation by cell density (Zhou et al, 2009). Thus, it is likely that H-89 increases the HM phosphorylation in LATS by activating LATS HM kinase.

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2nd Editorial Decision 04 April 2013

Thank you for the re-submission of your manuscript to The EMBO Journal and please accept my apologies for the delay in responding but we have just received the comments from the referees. Your study was sent to two of the original reviewers, who now consider that your manuscript is basically ready for publication. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few more minor details have been addressed, as follows.

Browsing through the manuscript myself I have noticed that the micrographs in figures 2D, 5B/F/G, S4B, S10 and S11 lack scale bars, which we require for clarity. In addition, the statistical analysis of your results is not properly described. As a guide, statistical analyses must be described either in the Materials and Methods section or in the legend of the figure to which they apply and will include a definition of the error bars used and the number of independent experiments performed, which in your case is not stated for each graph presented. Keep in mind that duplicates are not considered independent experiments. If the number of independent experiments is less than three, use of error bars is not appropriate and one representative experiment should be provided, clearly indicating this fact. I would like to draw your attention in particular to figure 5, where it is unclear whether your definition of error bars applies to all graphs, and figure S10, where the error bars are not defined.

As a novel initiative in The EMBO Journal, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Although optional at the moment, would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this initiative do not hesitate to contact me.

Thank you very much for your patience and congratulations in advance on a successful publication. Once these minor changes suggested are incorporated into the manuscript, you will receive an official acceptance letter with further instructions on how to proceed with the publication process.

REFEREE REPORTS

Referee #2

The authors have satisfactorily answered almost all of the reviewers' concerns. The reviews were all comprehensive and addressing these concerns required not only clarification but also extensive new experimental data. They have done this well and I believe that this manuscript is a significant contribution that warrants publication in the EMBO J.

Referee #3

The authors addressed my concerns.

2nd Revision - authors' response 09 April 2013

In brief, we have added scale bars in all our micrographic images and indicated their lengths in the respective figure legends; figure 2D, figure 5B/F/G, figure S4B, S10 and S11. We described the statistical analyses, number of independent experiments and definition of error bars in the figure legends for figure 5 and figure S10. Figure 5D was performed twice independently; therefore, we excluded error bars and indicated that it is a representative result of two independent experiments. Number of independent experiments for figure 5H/I and figure S10 are indicated in the materials & methods section.

We hope that our modified manuscript is now acceptable for publication in *EMBO J*. and we thank you again for your positive consideration.

