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Arhgef7 Promotes Activation of the Hippo pathway core kinase, Lats.

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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.)

Editor: Thomas Schwarz-Romond

1st Editorial Decision 26 June 2014

Thank you once again for your interest in The EMBO Journal and submitting the paper on potential contributions of beta-PIX towards Hippo-signalling for consideration to The EMBO Journal.

I received two sets of referee comments with one being, at least at the surface, being supportive. Careful reading of his/her comments reveals the currently rather preliminary status of analysis, a conclusion further corroborated by the much critical and elaborate comments from the second referee (needless to say both are, similar to you, recognized and trusted experts on the topic).

Following our referee cross-commenting practice, I further consulted with the refs to elucidate potential further pursuit. I am afraid to say, that in the absence of definitive mechanistic and further reaching functional corroboration (please refer to the explicit comments from ref#2 with regard to preliminary mechanistic insights as well as the choice of cell-line as to support actual functional

roles within Hippo signalling) would be needed before being able to offer further consideration. Given that this would also entail significant further experimental undertaking, we believe it would be in your best interest to return the paper to you at this point as to enable rapid consideration at a possibly less demanding title.

Please trust that I am really sorry to be unable to communicate more encouraging news, as we recognize the principle interest AND potential in these currently much too preliminary results. Please also understand that we have to ensure editorial consistency in our handling and I therefore hope that our rapid and fairly argued/transparent decision making might convince you to consider The EMBO Journal for submission of future studies from your lab.

REFEREE REPORTS:

Referee #1:

The manuscript by Heidary et al identifies bPIX as a novel mediator of Hippo pathway signaling. They show that bPIX binds to YAP and LATS kinases and is required for phosphorylation and repression of YAP and TAZ using multiple in vitro assays and cell lines.
The presented data are of good quality and the paper is well written and easy to follow. Conclusions follow from the data and I have only one small comment: The authors argue that bPIX acts downstream of cell density sensing and actin cytoskeletal rearrangements. However, this conclusion implies that the activity of bPIX is regulated by cell density and the actin cytoskeleton, which the authors do not show. Rather, the data only show that bPIX is required for the regulation of YAP by cell density and the actin cytoskeleton. I suggest changing the wording of the text accordingly.

Referee #2:

The Hippo tumor suppressor pathway plays important roles in linking proliferative control to cell-cell contact. The core of the Hippo pathway is a protein kinase cassette including Mst/Hippo kinases and the LATS kinases. The tumor suppressor NF2 recruits LATS kinases to the membrane where they are activated by MST kinases. LATS kinases in turn regulate the transcriptional coactivators YAP and TAZ to control cell proliferation and survival. Activation of the pathway has been linked to events at the cell membrane, including cell contact and cytoskeletal regulation. Various cancers have been associated with loss of the inhibitory effects of this kinase cascade. In light of the importance of the Hippo pathway in cancer understanding the mechanisms that regulate the MST and LATS kinases and their activation at the cell membrane are of interest. In this report, Arash et al explore the role of Arhgef7/ Pix in Hippo pathway activity.

Fig 1 presents identification of Pix as a TAZ interacting protein and confirms the interaction by co-IP with epitope-tagged proteins. siRNA knockdown of Pix is shown to increase nuclear localization of YAP in contact inhibited cells and to increase expression of YAP/TAZ transcriptional target RNAs. The data are of good quality and support the conclusions.

Disruption of the actin cytoskeleton with various inhibitors has been reported previously to reduce nuclear YAP/TAZ activity. Fig 2B shows that these effects are prevented by Pix knockdown. The

data in 2B-D are of good quality and support the conclusions.

Fig 2E shows overexpression of Pix and is interpreted as showing increased cytoplasmic retention of YAP. The level of YAP seems lower overall in the Pix-expressing cell. I don't see an increase in the amount of cytoplasmic YAP compared to adjacent cells. Quantification of multiple examples seems to be needed.

Fig 3B tests a C-terminally truncated form of Pix, which lacks GEF activity. This protein appears to produce a stronger effect on YAP localization (compared to fig 2E). The data in fig 3C, D shows that depleting the Pix GEF targets, CDC42 and Rac, does not increase YAP activity, consistent with previous reports. While these data are consistent with the suggestion that GEF activity of Pix may not be required for regulation of YAP, they fall short of making a strong case for this. Further work is needed to support this conclusion. Point mutations that disrupt GEF activity without compromising overall protein organisation would add confidence in the conclusion.

Figure 4A shows that Pix knockdown reduced phosphorylation of YAP on S127, consistent with reduced LATS kinase activity on YAP. 4B/C shows that Pix knockdown reduce basal YAP phosphorylation as well as following hippo pathway activation by latrunculin and other cytoskeletal inhibitors previously shown to activate the hippo pathway. The data are of good quality and support the conclusions.

Figure 5A shows interaction between epitope-tagged Pix and LATS1 in co-IP. 5C shows an increase in the recovery of epitope-tagged YAP bound to epitope-tagged LATS in the presence of Pix. While this is consistent with a 3-way interaction between the proteins, it is not sufficient evidence on which to base a key conclusion. A robust test of the hypotheis would be to ask if Pix knockdown compromised intraction between endogenous YAP and LATS.

Interestingly, LATS kinase activity appeared to be lower in Pix knockdown, based on LATS autophosphorylation. Lower intrinsic LATS activity would be a good explanation for the effects they reported above on YAP localization. Unfortunately, the authors don't examine this any further. This is problematic because it is a plausible alternative mechanism to explain the biological outcome of Pix knockdown.

Instead, Figure 6 goes on to map interaction domains with overexpressed YAP and LATS in co-IP experiments based on Pix deletion variants. All of the forms of Pix shown bind YAP, albeit with differing apparent efficiency. A similar problem exists for the interaction of the Pix deletions with LATS. The data are overinterpreted, in my opinion. Figure 7 shows that expression of the C-terminally truncated forms of Pix have less effect on YAP nuclear localization in cells than the full length protein. The N-terminal truncation appears to behave like full length in being able to reduce nuclear YAP.

Figure 8 shows that Pix overexpression in MDA-MB-231 cells increases cytoplasmic retention of YAP. Pix overexpression also compromised migration in a scratch assay and reduced proliferation. Controls to confirm that the effect of Pix overexpression is actually due to reduced YAP activity are needed.

MDA-MB-231 cells are a surprising choice for these experiments. MDA-MB-231 cells are mutant for NF2 (Dupont et al 2011), so YAP and TAZ are uncoupled from regulation by the Hippo pathway at the membrane (NF2 promotes interaction between MST and LATS kinases, potentiating LATS

activation). Regulation of YAP/TAZ by the actin cytoskeleton is intact in these cells. Cell lines with an intact Hippo pathway might be a better choice for these experiments.

The authors interpret their data as evidence that Pix acts as a scaffold promoting interaction between LATS and YAP/TAZ. This conclusion is consistent with some of the data, but is not unambiguously supported by the experiments shown. Considerably more work would be needed to provide robust support for the proposed mechanism.

Additional Author Correspondence 26 June 2014

First, I wanted to thank you for the prompt review. I was wondering whether you would be willing consider a revised manuscript. We already have data in hand to address several of the concerns raised and along with ongoing experiments can strengthen our mechanistic model and functional relevance in a timely fashion. If necessary, I can provide an outline of what we can provide to address the reviewers comments.

Additional Editorial Correspondence 26 June 2014

As indicated, the paper has significant potential. On the other hand, it would have to be taken to a significantly higher level with regard to the cellular system (as ref#2 mentioned, these cells were simply not appropriate to make the claim that betaPIX would contribute to bona-fide Hippo-signaling) as well as definitive molecular understanding: definitively ruling in/out GEF-activity and determining how it would control kinase activity that should be assessed also beyond autophosphorylation. Together with establishing functional causality (loss of betaPIX, respective loss of its molecular activity) in some physiological context, would be necessary.

In case you were able to provide this in a relatively timely manner, I would indeed be happy to first of all assess it myself at the editorial level.

Resubmission 03 October 2014

Response to Referees' Comments

Referee #1

The manuscript by Heidary et al identifies bPIX as a novel mediator of Hippo pathway signaling. They show that bPIX binds to YAP and LATS kinases and is required for phosphorylation and repression of YAP and TAZ using multiple in vitro assays and cell lines. The presented data are of good quality and the paper is well written and easy to follow. Conclusions follow from the data and I have only one small comment: The authors argue that bPIX acts downstream of cell density sensing and actin cytoskeletal rearrangements. However, this conclusion implies that the activity of bPIX is regulated by cell density and the actin cytoskeleton, which the authors do not show. Rather, the data only show that bPIX is required for the regulation of YAP by cell density and the actin cytoskeleton. I suggest changing the wording of the text accordingly.

We thank the reviewer for the positive comments that the data is of good quality, the paper well written and that our conclusions follow from the data. As requested, we have modified the wording of the text to indicate that βPIX is required for regulation of YAP by cell density and actin cytoskeleton rather than saying it functions downstream of these signals.

Referee #2

We thank the reviewer for pointing out that the data presented in Figure 1, 2 and 4 is "of good quality and support the conclusions". The reviewer indicated that there were a few issues in the remaining figures that should be addressed. We thank the reviewer for pointing out ways to strengthen our conclusions and we have incorporated the suggestions into the updated manuscript as detailed below.

Fig 2E shows overexpression of βPix and is interpreted as showing increased cytoplasmic retention of YAP. The level of YAP seems lower overall in the βPix-expressing cell. I don't see an increase in the amount of cytoplasmic YAP compared to adjacent cells. Quantification of multiple examples seems to be needed.

A representative image is shown in this figure. This type of experiment was quantified in the context of analyzing WT and mutant versions of βPIX later in the manuscript in Figures 3 and 6. We now refer to this quantitation in the text. These results show that there is enhanced cytoplasmic levels of YAP that yields an even distribution between cytoplasm and nucleus (rather than exclusive cytoplasmic localization). We now clarify this in the text by stating that overexpression of βPIX results in a relocalization of Yap/Taz such that it is evenly distributed in the cytoplasm and nucleus whereas in neighboring, non-transfected cells Yap/Taz is predominantly nuclear.

Fig 3B tests a C-terminally truncated form of βPix, which lacks GEF activity. This protein appears to produce a stronger effect on YAP localization (compared to fig 2E). The data in fig 3C, D shows that depleting the βPix GEF targets, CDC42 and Rac, does not increase YAP activity, consistent with previous reports. While these data are consistent with the suggestion that GEF activity of βPix may not be required for regulation of YAP, they fall short of making a strong case for this. Further work is needed to support this conclusion. Point mutations that disrupt GEF activity without compromising overall protein organisation would add confidence in the conclusion.

We agree this is important and, as suggested, we generated a GEF-inactive mutant of βPix by introducing two previously described point mutations (L238R/L239S). The results show that transient overexpression of this mutant retains the ability to relocalize Yap/Taz to the cytoplasm, similar to WT, thereby strengthening our conclusion that Cdc42/Rac1 activity is dispensable for βPIX function in regulating Yap/Taz (Fig. 3A).

Figure 5A shows interaction between epitope-tagged βPix and LATS1 in co-IP. 5C shows an increase in the recovery of epitope-tagged YAP bound to epitope-tagged LATS in the presence of βPix. While this is consistent with a 3-way interaction between the proteins, it is not sufficient evidence on which to base a key conclusion. A robust test of the hypotheis would be to ask if βPix knockdown compromised intraction between endogenous YAP and LATS.

As suggested by the reviewer, we examined the effect of βPix knockdown on the magnitude of the interaction between endogenous Yap and Lats. We now show (Fig. 6C) that siRNA-mediated loss of βPIX in NMuMG cells attenuates the interaction between endogenous Yap and Lats. This is in line with our overexpression experiment, which shows an enhanced interaction of Lats with Yap in the presence of βPix. Moreover, we have also added new data to show that unlike WT βPIX, overexpression of a βPIX mutant (ΔKER) that does not bind Yap or Lats, does not enhance their interaction (Fig. 6B). Altogether, these data provide strong evidence in support of our model that βPIX acts as a scaffold for Lats and Yap/Taz.

Figure 8 shows that βPix overexpression in MDA-MB-231 cells increases cytoplasmic retention of YAP. βPix overexpression also compromised migration in a scratch assay and reduced proliferation. Controls to confirm that the effect of βPix overexpression is actually due to reduced YAP activity are needed. MDA-MB-231 cells are a surprising choice for these experiments. MDA-MB-231 cells are mutant for NF2 (Dupont et al 2011), so YAP and TAZ are uncoupled from regulation by the Hippo pathway at the membrane (NF2 promotes interaction between MST and LATS kinases, potentiating LATS activation). Regulation of YAP/TAZ by the actin cytoskeleton is intact in these cells. Cell lines with an intact Hippo pathway might be a better choice for these experiments.

As mentioned by the reviewer, in MDA-MB-231 cells the Hippo pathway is uncoupled from YAP/TAZ thereby resulting in constitutive nuclear localization of YAP/TAZ. It is exactly for this reason that we selected these cells, as it allowed us to address the question of whether increasing the expression of βPIX, which we propose acts at the level of Lats, can re-engage the Hippo kinase cassette to attenuate Yap/Taz nuclear activity. We have clarified the rationale for selecting these cells in the text and have added additional supportive data. In the previous version of the manuscript we showed that stable expression of βPIX inhibits cell proliferation and migration. We now show that loss of YAP and/or TAZ similarly inhibits proliferation and migration (Suppl. Fig. 3), consistent with the notion that YAP/TAZ are required for these tumor-promoting properties. Moreover, we used siRNAs to abrogate the expression of LATS1 and LATS2 in control MDA-MB-231 cells, and demonstrate that this results in a further increase in nuclear YAP/TAZ and target gene expression. Thus, LATS1/2 are present in these cells and retain some activity towards YAP/TAZ. Importantly, we go on to show that abrogation of LATS1/2 expression in βPIX-expressing cells prevents the βPIX-induced relocalization of YAP/TAZ to the cytoplasm and βPIX-mediated inhibition of target gene expression. These results now clearly show that increased expression of βPIX recouples YAP/TAZ to the hippo kinase cassette. We don't believe these results contradict prior publications as they might suggest that βPIX functions downstream of NF2 to promote regulation of YAP/TAZ by Lats.

We thank the reviewers for their comments and feel that in addressing them we have greatly strengthened the manuscript and our model that βPIX acts as a scaffold for Lats and Yap/Taz to promote Lats-mediated phosphorylation and inactivation of the transcriptional activity of Yap/Taz.

2nd Editorial Decision 09 October 2014

One of the original referees commented on your reworked manuscript. As you will see from the enclosed remarks, s/he essentially endorses publication of the study while emphasizing that some potential valuable information was removed from the dataset, seemingly to overall focus the message.

Based on these comments, I am prepared to initiate necessary steps for formal acceptance:

(i) For production, we would need high quality, individual images (please see below for detailed instructions.

(ii) Please notice that The EMBO Journal encourages submission of source data, as to increase data reliability and reproducibility. We would kindly ask for a single PDF for every figure representing uncropped/unprocessed gels (and minimally for those that support the main conclusions) of the paper. In case of graphs/data-quantification, we also offer to host the underlying excel sheets as supporting information. (This initiative voluntary for the moment. Your contribution would however be much appreciated.

On this note, we realized during our analyses that the DAPI panels in figure 2B, DMSO controls, seem to have been swapped accidently.

(iii) Please provide a 2-up to 4 bullet point SYNOPSIS that emphasizes the major advance provided by your study. Short and concise terms would be appreciated (either as separate file or via email).

(iv) We also offer graphic featuring of the study. A 'graphical abstract' of the size 550x150(max 400) pixels (model of bPIX scaffolding function?) beyond the sketch in the current paper?) would facilitate readers perception.

I hope that this message will be received as good news and would be grateful for your timely attention and response as to facilitate rapid production/publication.

I take the liberty to congratulate you already on this occasion to a fine study!

REFEREE REPORT:

Referee #1:

The authors have addressed some of my specific requests for additional data. In particular the siRNA experiment showing the depletion of bPIX reduces interaction between ~LATS and YAP adds strength to their hypothesis. I was disappointed to see that they have ignored the suggestion pertaining to the alternative mechanism, reduced LATS activity, which was equally well supported in the original version of the manuscript. It appears that some of the relevant data have been removed from this version. It is evident that the authors favor one mechanism, but they do not do themselves a service by ignoring the alternative. That said, the new data make the manuscript publishable.