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Role of Angiomotin-like 2 mono-ubiquitination on YAP inhibition

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Transaction Report:

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

1st Editorial Decision 29 June 2015

Thank you for your submission to EMBO reports. We have now received reports from the two referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although both referees find the topic of interest and in principle suitable for us, referees 1 especially raises a number of technical issues, and considers that further experiments are needed to provide convincing support for your model of the role of USP9X regulation of YAP through control of Amot monoubiquitylation.

All the issues raised by referee 1 are pertinent and should be addressed. In addition, we fully agree with the concern of referee 2 regarding the physiological relevance of the study. If there is a way to address this issue within a reasonable time-frame by adding some in vivo work, these results would dramatically bolster the significance of your study. Including such data, however, would not be a precondition for the acceptance of your study.

Please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

Revised manuscripts must be submitted within three months of a request for revision unless previously discussed with the editor; they will otherwise be treated as new submissions.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines

(http://embor.embopress.org/authorguide#revision)
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
- a separate PDF file of any Supplementary information (in its final format)

In addition, EMBO reports now accommodates the inclusion of extra figures (up to five) in the online version of the manuscript. These are presented in an expandable format inline in the main text so that readers who are interested can access them directly as they read the article. They are also provided for download in a separate typeset PDF to accompany the Article PDF. These should be those of particular value to specialist readers, but which are not required to follow the main thread of the paper (and not additional controls or reagent optimization). These should be labeled expanded view, and the rest supplementary.

We also encourage the publication of original source data -particularly for electrophoretic gels and blots, but also for graphs- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures and an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, please contact me if I can be of any assistance.

REFEREE REPORTS

Referee #1:

In this manuscript, Dr. Lim and his colleagues reported a new link between the deubiquitinase USP9X and AMOTL2, through which USP9X regulated YAP phosphorylation, localization, transcriptional activity and its function in cell proliferation. Mechanistically, USP9X promoted YAP activity through associating with and deubiquitinating AMOTL2 and subsequent reduction of AMOTL2-LATS2 interaction and LATS2 kinase activity to YAP. This work is potentially significant as it uncovered a new mechanism of ubiquitinaon/deubiquitination of AMOTL2 in the regulation of the Hippo signaling pathway. However, I do have a few concerns on the role of USP9X uncovered by this study:

1. The authors claimed mono-ubiquitination of AMOTL2 is the major ubiquitinated species for its inhibitory effect on YAP activity. To my knowledge, I think the presented data is not enough to support the author's claim. I would like to see the ubiquitinated pattern of AMOTL2 by mutant ubiquitin (K free) parallel to Fig 4A and its role on YAP parallel to Fig 6E.
2. USP9X is required for maximal YAP activity from knock-down assays in sparse condition (Fig 2A-C). Could USP9X overexpression in densely cultured cells reactivate YAP (such as phosphorylation, localization) as in sparsely cultured cells? Could USP9X expression decrease by cell density increasing? Based on Fig 2C, the protein level of USP9X is increased from sparse to dense conditions (compare the control panels in Fig 2C). If not, please discuss the possible reasons.
3. As USP9X is recovered as a YAP-associating protein (Supplementary table 1) and USP9X formed an immunoprecipitation complex with AMOTL2 (Fig 4E-F). The authors didn't provide any data to mention the interaction between YAP and USP9X. Please give me an explanation.
4. Ubiquitination of AMOTL2 seems to be important for the interactions between AMOTL2 and YAP, andAMOTL2 and LATS2. Does AMOTL2-mediated regulation of USP9X on LATS2 activity require YAP's association with AMOTL2?

Referee #2:

The authors identify a novel ubiquitylation mechanism for regulating AMOT - like proteins. They show that USP9X is a DUB for AMOTL2 using a variety of biochemical and cell culture approaches. What is lacking is any in vivo genetic evidence for an important role for USP9x. Nevertheless, if this mechanism proves to be important in vivo, this paper will be cited widely and will be an important contribution to the field.

Specific points:

1. I found the model in Fig7 a little confusing. Perhaps just show wild-type versus USP9X mutant or overexpressing cells.

1st Revision - authors' response 28 September 2015

We would like to thank the reviewers for insightful and productive suggestions. Accordingly, we have performed the suggested experiments.

In addition to reviewers' comments, we also have added new data in Appendix Fig S3, which shows that USP9X IP-complex can de-ubiquitinate AMOTL2 *in vitro*. We believe that this result further strengthens our conclusion that AMOTL2 is a substrate of USP9X.
Below, we describe our new experiments and responses to the reviewers' comments.

Referee #1:

In this manuscript, Dr. Lim and his colleagues reported a new link between the deubiquitinase USP9X and AMOTL2, through which USP9X regulated YAP phosphorylation, localization, transcriptional activity and its function in cell proliferation. Mechanistically, USP9X promoted YAP activity through associating with and deubiquitinating AMOTL2 and subsequent reduction of AMOTL2-LATS2 interaction and LATS2 kinase activity to YAP. This work is potentially significant as it uncovered a new mechanism of ubiquitination/deubiquitination of AMOTL2 in the regulation of the Hippo signaling pathway. However, I do have a few concerns on the role of USP9X uncovered by this study:

1. The authors claimed mono-ubiquitination of AMOTL2 is the major ubiquitinated species for its inhibitory effect on YAP activity. To my knowledge, I think the presented data is not enough to support the author's claim. I would like to see the ubiquitinated pattern of AMOTL2 by mutant ubiquitin (K free) parallel to Fig 4A and its role on YAP parallel to Fig 6E.

This is a very insightful comment and we thank the reviewer for pointing out this issue. To address the reviewer's concern, we have performed new experiments as suggested.
First, we performed ubiquitination assay for AMOTL2 using either WT Ub or K0 mutant Ub (K free). We found that both WT and K0 Ub are equally incorporated into AMOTL2. This new result is provided in the newly revised Fig 5A.

Second, we performed LATS2 kinase assay using either WT Ub or K0 Ub. As expected, co-transfection of AMOTL2 increased LATS2 kinase activity, and addition of WT Ub further increased LATS2 activity. Importantly, K0 Ub also showed comparably increased LATS2 activity to WT Ub. This result is provided in Appendix Fig S4. Taken together, experiments using K0 Ub indicate that AMOTL2 mono-ubiquitination is functionally sufficient.

2. USP9X is required for maximal YAP activity from knock-down assays in sparse condition (Fig 2A-C). Could USP9X overexpression in densely cultured cells reactivate YAP (such as phosphorylation, localization) as in sparsely cultured cells? Could USP9X expression decrease by cell density increasing? Based on Fig 2C, the protein level of USP9X is increased from sparse to dense conditions (compare the control panels in Fig 2C). If not, please discuss the possible reasons.

This is another insightful suggestion. First, we would like to note that efficient over-expression of USP9X was technically somehow difficult, possibly due to its large size (>200kD) for a retroviral packaging or toxicity arising from stably too much over-expression. Therefore, we first addressed this issue by transfecting RPE cells with USP9X-V5 WT/CS (catalytically inactive mutant) and detecting transfected cells by V5 immunostaining. As a control, we transfected RPE cells with EGFP and detected EGFP positive cells by the same method. We co-immunostained YAP and analyzed YAP localization in each condition (cells were in dense condition). We observed that YAP is diffusely localized throughout the cell in untransfected, EGFP transfected and USP9X-CS transfected cells. However, cells transfected with USP9X-WT showed clear nuclear localization of YAP. We provide these results in Fig 2D and E. Next, we attempted to establish stable cells expressing USP9X WT or CS mutant. As a consequence, we successfully obtained two independent RPE clones. Using these cell lines, we demonstrate that increased expression of USP9X WT, but not CS mutant, increases nuclear YAP when seen by cell fractionation (Fig 2F), increases YAP target gene levels (Appendix Fig S1A), and decreases YAP S127 phosphorylation (Fig 3B).

We also took the advantage of recently described CRISPR-SAM system [1]. This system utilizes nickase-deficient Cas9 fused to transcriptional co-activators. Introduction of sgRNA targets Cas9 to target genes. We obtained three sgRNAs that successfully increased USP9X level in RPE cells. Using them, we demonstrate that USP9X over-expression increases nuclear YAP when seen by cell fractionation (Fig 2G), increases YAP target gene levels (Appendix Fig S1B), and decreases YAP S127 phosphorylation (Fig 3C). Importantly, we confirm that knock-down of USP9X by siRNA reduces YAP target gene levels back, excluding off-target effects of sgRNAs (Appendix Fig S1C). Taken together with immunostaining results, these data clearly show that gain of USP9X function can re-activate YAP in densely growing cells.

We also consider potential regulation of USP9X by cell-density as an important issue. Although Western blot results obtained from MCF10A cells (Figs 1C and 3A) show some increment of USP9X in dense conditions, we never observed such phenomenon in RPE cells. We do not clearly understand the reason underlying such cell type dependency yet. However, whatever the mechanism is, we believe that regulation of USP9X at the protein level is not a general mechanism. We rather speculate that USP9X activity (also potentially protein level) might be regulated by LATS-mediated phosphorylation. This conjecture is based on the fact that LATS2-USP9X interaction increases in okadaic acid treated cells [2], in which LATS2 is activated, and that USP9X contains putative LATS target sequence (Appendix Fig S5). We have described these in the discussion section.

3. As USP9X is recovered as a YAP-associating protein (Supplementary table 1) and USP9X formed an immunoprecipitation complex with AMOTL2 (Fig 4E-F). The authors didn't provide any data to mention the interaction between YAP and USP9X. Please give me an explanation.

We thank the reviewer for the insightful comment. As the reviewer pointed out, we first identified USP9X as a YAP immunoprecipitant. We also confirmed their interaction with over-expressed USP9X and YAP in 293T cells as well as with endogenous proteins in RPE and MCF10A cells (Now provided in Fig EV5). However, we believe that the YAP immunoprecipitated complex surely included both direct and indirect interacting proteins. After we started to investigate the functional role of USP9X in the Hippo signaling, we reached the conclusion that USP9X regulates Hippo signaling through de-ubiquitination of AMOTL2.

Currently, we think that YAP-USP9X interacts indirectly through YAP-LATS interaction. Supporting this view, Couzens et al. reported LATS2-USP9X interaction in okadaic acid treated cells [2], wherein LATS2 is activated. Importantly, our purification was also performed in detached cells, which activates LATS kinase. In addition, as we described in response to comment 2, USP9X has putative LATS2 target sequence (Appendix Fig S5). Taken together, we hypothesize that following LATS activation, both LATS-YAP and LATS-USP9X complexes are formed, likely leading to indirect interaction between YAP and USP9X. Of course, this hypothesis requires additional experimental validation and is an important future subject of research. We have described these in the discussion section.

4. Ubiquitination of AMOTL2 seems to be important for the interactions between AMOTL2 and YAP, and AMOTL2 and LATS2. Does AMOTL2-mediated regulation of USP9X on LATS2 activity require YAP's association with AMOTL2?

As the reviewer has pointed out, understanding the mechanistic relationship between AMOTL2-YAP interaction and other AMOTL2 modifications is a very important mechanistic question. To address this question, we generated AMOTL2 Y213A mutant (mutation in the PPXY motif), that fails to interact with YAP. Very interestingly, AMOTL2 Y213A mutant also failed to be ubiquitinated (Now provided in Fig 6E). Therefore, AMOTL2-YAP interaction is required for either recruitment of hypothetical E3 ligase or repulsion of USP9X. AMOTL2 is regulated by multiple mechanisms including AMOTL2-YAP interaction [3, 4], phosphorylation by LATS [5, 6] and mono-ubiquitination (Our study). Intriguingly, these regulatory mechanisms seem to cooperate in positive feed-forward manner. In the future, a systematic study using multiple AMOTL2 mutants would allow us to dissect the precise mechanism of AMOTL2 regulation. In addition to Fig 6E, we have described this in the discussion section, too. We are grateful for the reviewer's comments.

Referee #2:

The authors identify a novel ubiquitylation mechanism for regulating AMOT - like proteins. They show that USP9X is a DUB for AMOTL2 using a variety of biochemical and cell culture approaches. What is lacking is any in vivo genetic evidence for an important role for USP9x. Nevertheless, if this mechanism proves to be important in vivo, this paper will be cited widely and will be an important contribution to the field.

In this manuscript, we identified AMOTL2 as a target of USP9X by using both cellular genetics and biochemical approaches. We completely agree with the reviewer's comment. While it is practically not suitable to perform *in vivo* mouse genetic experiments during the revision period, we have performed additional functional assays regarding the importance of AMOTL2 ubiquitination.

In our previous manuscript, we only showed that AMOTL2 knock-down decreased YAP S127 phosphorylation and increased YAP target gene expressions, and that only AMOTL2 WT (but not AMOTL2 K347/408R mutant) rescues this. We further expand this result by performing functional assays including 1) analysis of EMT markers by RT-qPCR, 2) transwell migration assay and 3) soft agar assay. These results are provided in Fig 7.

We believe that addition of these new functional assays would reinforce our major finding: the importance of AMOTL2 ubiquitination as a novel regulatory mechanism of Hippo signaling. We are grateful to the reviewer for the insightful comment.

Specific points:

1. I found the model in Fig7 a little confusing. Perhaps just show wild-type versus USP9X mutant or overexpressing cells.

We agree with the reviewer's comment. According to the reviewer's suggestion, we have modified the model figure by comparing USP9X proficient and deficient situations. The new figure is provided in Fig 9. We are grateful for the reviewer's comment.

1. Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H*, et al.* (2015) Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* **517**: 583-8
2. Couzens AL, Knight JD, Kean MJ, Teo G, Weiss A, Dunham WH, Lin ZY, Bagshaw RD, Sicheri F, Pawson T*, et al.* (2013) Protein interaction network of the mammalian Hippo pathway reveals mechanisms of kinase-phosphatase interactions. *Sci Signal* **6**: rs15
3. Zhao B, Li L, Lu Q, Wang LH, Liu CY, Lei QY, Guan KL (2011) Angiomotin is a novel Hippo pathway component that inhibits YAP oncoprotein. *Gene Dev* **25**: 51-63

4. Wang WQ, Huang J, Chen JJ (2011) Angiomotin-like Proteins Associate with and Negatively Regulate YAP1. *Journal of Biological Chemistry* **286**: 4364-4370
5. Chan SW, Lim CJ, Guo F, Tan I, Leung T, Hong W (2013) Actin-binding and cell proliferation activities of angiomotin family members are regulated by Hippo pathway-mediated phosphorylation. *J Biol Chem* **288**: 37296-307
6. Hirate Y, Hirahara S, Inoue K, Suzuki A, Alarcon VB, Akimoto K, Hirai T, Hara T, Adachi M, Chida K*, et al.* (2013) Polarity-dependent distribution of angiomotin localizes Hippo signaling in preimplantation embryos. *Curr Biol* **23**: 1181-94

2nd Editorial Decision 15 October 2015

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the report of the referee that was asked to assess it, which is copied below.

There are only some editorial issues that remain to be done before we can proceed with the official acceptance of your study.

Every EMBO reports paper now includes a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version and they are freely accessible to all readers. You have already kindly provide a text, which I modified slightly. Please let me know if you do NOT agree with the suggest changes.

"This study reports a new regulatory mechanism of the Hippo signaling governed by mono-ubiquitination of AMOTL2. AMOTL2 mono-ubiquitination is required to bind and activate LATS kinase and to inhbit YAP activity and is regulated by the USP9X de-ubiquitinase."

This will be accompanied by a thumbnail image (part of Figure 9) and a Synopsis image (500 x 400 pixel) of your choice. Could you please provide a synopsis image?

REFEREE REPORTS

Referee #1:

The authors have addressed the concerns raised in my initial review.

2nd Revision - authors' response 16 October 2015

Authors made necessary editorial changes.

3rd Editorial Decision 20 October 2015

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.

