EMBO reports

UBTD1 is a mechano-regulator controlling cancer aggressiveness

Torrino S., Roustan F.R., Kaminski L., Bertero T., Pisano S., Ambrosetti D., Dufies M., Uhler J.P., Lemichez E., Mettouchi A., Gesson M. , Laurent K., Gaggioli C., Michiels J.F., Lamaze C., Bost F. & Clavel S.

Review timeline:

Submission date:	12 June 2018
Editorial Decision:	18 July 2018
Revision received:	7 November 2018
Editorial Decision:	17 December 2018
Revision received:	21 December 2018
Editorial Decision:	11 January 2019
Revision received:	18 January 2019
Accepted:	29 January 2019

Editor: Achim Breiling

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 18 July 2018

Thank you for the submission of your research manuscript to EMBO reports. We have now received the full set of referee reports that are copied below.

I am sorry to say that the decision on your manuscript is not a positive one. As you will see, although the referees feel the study is of interest, all three referees have several concerns, indicating that the study is presently too preliminary, that the conclusions are not sufficiently supported by the data, and that several controls are missing (in particular regarding the knock down efficiency in the RNAi experiments).

Given these comments, and considering the amount of work required to address them, we cannot offer to publish your manuscript. However, in case you feel that you can address the referee concerns in a timely and thorough manner, and can obtain data that would considerably strengthen the study as outlined above and in the referee reports, we would have no objection to consider a new manuscript on the same topic in the future. Please note that if you were to send a new manuscript this would be assessed again with respect to the literature and the novelty of your findings at the time of resubmission.

I am sorry to have to disappoint you this time, and I thank you once more for your interest in our journal.

REFEREE REPORTS

Referee #1:

In this manuscript, the role of UBTD1 is examined and is shown to affect the mechanical properties of cancer cells, regulates YAP protein levels and promotes with tumorigenic properties in cells and is correlated with disease status in prostate and lung cancer patients.

1. Activation of the Hippo pathway leads to LATS-mediated phosphorylation of YAP, which is followed by ubiquitination and degradation of Yap. Here, it is shown that siUBTD1 leads to an increase in Yap protein levels through the ability of UBTD1 to recruit beta-TrCP and the ubiquitination machinery to YAP. Assuming that the experiments can be reproduced with the appropriate controls (see below), the results showing that YAP levels increase upon loss of UBTD1 are convincing. However, the authors' conclusion that UBTD1 functions independent of Hippo, is not sufficiently supported by the very weak data provided (see specific comments below). This is not a key point of the paper, and either Hippo-dependent or independent function would be equally interesting, but if the authors wish to make the point of Hippo-independent, additional lines of experimental evidence are required to support this statement.

2. The majority of the conclusions drawn rely heavily on the use of siUBTD1. Thus, it is absolutely essential that the knockdown efficiency be shown in every (or at least the majority of panels) in order to draw the conclusion that the observed effects are at least correlated with loss of expression of UBTB1 (as opposed to off-target effects). Disturbingly, knockdown efficiency by PCR is not shown anywhere in the manuscript (main or EV) and while a reduction in UBTD1 protein by immunoblotting was shown in 2 panels (Fig 1b and 3b), these data were for rather minor points. The authors have done qPCR and blots for many of the experiments reporting the effects of siUBTD1, thus there is no reason why the knockdown could not be shown in parallel. It would not be sufficient to add data showing the knockdown efficiency in a separate conducted experiment as part of a rebuttal, rather all of the key experiments would need to be repeated.

3. It is known that siRNAs can have off-target effects, thus it is important to test key points with multiple siRNAs (ie deconvolve the pool used in the study). Rescue experiments with siResistant UBTD1 is also a standard requirement for siRNA experiments. Alternative knockdown approaches would also be valuable (CRISPR, shRNA) though not essential.

4. The authors should confirm the specificity of the UBTD1 antibody in IF experiments under both soft and firm ECM conditions (where changes in localization are observed) and in both cell lines using siRNAs.

5. Fig. 2f and 3H. PLA experiments. The authors should include at least one negative control to confirm that the signal is specific (such as siUBTD1, and/or results when only one of the PLA protein detection reagents is present).

6. Fig. 3A. The authors indicate that 'As expected, upon increased cell density, YAP level increased and YAP proteins were phosphorylated on ser127. In the blots shown, there actually is no increase in YAP protein levels in DU145. In some cell lines a decrease in YAP/TAZ protein levels are observed upon increased cell density due to phosphorylation/degradation.

7. Fig. 3B. The authors state that level of YAP phosphorylation (P-ser127YAP/total YAP ratio) remained unchanged and reference Fig 3B and Fig EV3A. While the blot does show there is no increase in the level of p-YAP upon siUBTD1 (rather it seems that there is less), but since total YAP increases, one would conclude that the relative levels of phosphorylated YAP to total YAP actually decrease. The authors should quantitate this to provide support for their conclusion, that contrasts what is evident in the blot. What cell density was used as compared to the densities examined in Fig. 3A?

8. Fig. 3C. To conclude that LATS/MST phosphorylation is not altered by siUBTD1, a positive control to confirm phosphorylation of LATS and MST can be efficiently detected in these cell lines is required (ie could try low vs high density). Moreover, since the results are negative, it is essential to confirm loss of UBTD1 protein by blotting aliquots in parallel. It would be useful to indicate which phospho-sites are being monitored in Mst/Lats (the methods simply indicate a catalog number) so it would be helpful to add this information, preferably in the figure but minimally in the methods.

9. The authors neglected to include the data they referred to showing that "less PLA between YAP and UBTD1 when UBTD1 is localized at the cell-cell contact sites instead of the cytoplasm."

10. Fig. 3J. The knockdown efficiency of siUBTD1 must be shown (in this and all other knockdown experiments examining target gene expression).

11. One conclusion of the data in Figure 3 is "that UBTD1 depletion is sufficient to induce a major upregulation of YAP signaling, independently of the Hippo pathway." Additional lines of experimental evidence are required to support this statement.

12. Fig. 4a: Does loss of YAP prevent siUBTD1-mediated EMT?

13. In Fig. 4, the authors show that siUBTD1 promotes various tumorigenic properties of cells. It would be important to show that the observed effects are dependent on YAP as opposed to a role for UBTD1 that is independent of YAP. A more balanced consideration of this possibility would enhance the study.

I4. In the patient samples, is there a correlation between UBTD1 and Yap nuclear localization and/or target gene expression?

15. Other points of curiosity:

Does overexpression of UBTD1 decrease YAP expression and/or cause relocalization?
Does UBTD1 also act on WWTR1/TAZ?

Referee #2:

The manuscript by Torrino et al. outlines a relationship between UBTD1 and mechanotranduction in the prostate and lung cancer cell lines. The authors report that UBTD1 localization is impacted by cytoskeletal changes induced by mechanical stimuli and that UBTD1 levels mediate cell tractions forces, RhoA activation, and YAP activity. The authors further report that prostate and lung cancer tissues show reduced UBTD1 levels, and that cancer patients with low expression of UBTD1 in their biopsies have poorer long-term survival.

UBTD1 is a ubiquitin-domain protein that has not been well studied, and this manuscript is one of few that investigates the biological roles of this protein. This manuscript therefore reports findings that are important and interesting. The relationship with cytoskeletal remodeling and mechanotransduction is of particular interest given the increasing interest in understanding these signals in the context of disease.

However, many of the conclusions made about UBTD1 molecular function are preliminary and need further support before the manuscript is suitable for publication. In particular, the mechanisms linking UBTD1 to YAP regulation are poorly supported by the presented data. Several comments are outlined below that need to be addressed.

Comments:

- Many conclusions about UBTD1 localization are made by IF microscopy using an UBTD1 antibody. The authors need to validate that the UBTD1 staining shown is specific. This should be very easy given how efficient the knockdown is following siRNA treatment of the cells.

- The data relating junctional localization of UBTD1 and YAP localization is confusing. Some data, such as the data in Fig 1F, suggest that junctional UBTD1 localization correlates with nuclear YAP localization. Other data, such as the data in Figure EV1E, shows that junctional UBTD1 localization correlates with conditions that promote cytoplasmic YAP localization. Further, the model shown in Fig 5 is confusing with respect to YAP localization and activity. If YAP regulation remains central to the authors conclusions about UBTD1, the authors need to provide a clear relationship between the factors with more much more insight into the molecular mechanisms impacting YAP localization and activity.

- The reported increased levels of YAP with cell density that is shown in Fig 3A is surprising based

on what has been reported about cell density/Hippo-mediated regulation of YAP. The authors should quantify the total levels of YAP in this experiment normalizing to an appropriate control. Also, by eye the levels of p-YAP shown in the western blot in Fig 3B appear to be reduced relative to the total YAP shown, and the total Yap may not be changing when taking into account the loading controls. The authors should quantify the p-YAP levels and recheck the quantitation of the total YAP normalized to the loading controls. If the authors do see a decrease in p-YAP, this should be reported in the manuscript.

- The authors make statements about UBTD1 impacting YAP independent of Hippo pathway signaling without actually testing whether any observed effects are indeed independent of Hippo pathway signaling. In fact, contrary to what is stated in the manuscript, the images presented in Fig 3B appear to show that phospho-YAP levels are changing upon UBTD1 knockdown, suggesting that the LATS-mediated phosphorylation of YAP is changing. The authors should recheck their quantitation and if Hippo-independent statements are to be made further experiments, experiments in the absence of LATS1/2 need to be performed.

- Given the effects on UBTD1 by cell density the authors need to state the confluence conditions that were used for each experiment.

- The conclusion in the manuscript related to YAP degradation being regulated by UBTD1 is very poorly supported by data. Given the central importance of this mechanism to the manuscript much more data showing an impact on YAP stability is needed. At a minimum, experiments that examine YAP protein turnover are required.

- If indeed YAP ubiquitination is regulated by UBTD1, it is unclear how the authors believe UBTD1 participates in YAP ubiquitination. Based on the data shown in Figs 3F and 3G UBTD1 and YAP stably interact, which is generally not the case for ubiquitin-regulating proteins that promote degradation. More mechanistic insight into the roles of UBTD1 in YAP ubiquitination is required. How do the authors rationalize why a ubiquitin-like protein acts to promote polyubiquitin modification of YAP? If the authors believe that UBTD1 acts as an adaptor for beta-TRCP and/or UBCH5c, as drawn in the model in Fig 5, the authors should perform experiments to prove this. Alternatively, if the authors believe that the enzymatic activity of UBCH5 is affected by UBTD1, experiments to show such activity need to be included.

- The PLA data is not very convincing and is lacking several controls. No antibody controls are shown and often very few spots are detected. Ideally, each of the PLA experiments should be performed following UBTD1 knockdown to show that the observed signals are indeed representing interactions.

- The experiment in Fig 3E does not properly address whether UBTD1 regulates YAP ubiquitination, and the conclusions made by the authors are not supported by the presented data. First, the data as presented cannot exclude differences in the transfection of the His-Ub in the different samples. Also, often experiments examining direct modification of a protein by Ub in cells include a second IP to ensure the specificity of the protein being detected. If the authors propose that UBTD1 is part of the YAP ubiquitination complex, this experiment should be performed in vitro with purified components to prove this role for UBTD1.

- It is unclear why the total YAP levels in Fig 3E are not increased with UBTD1 knockdown. This data is inconsistent with the ubiquitin levels shown in the same figure and the conclusions made by the authors from prior figures.

- There is no validation that UBTD1 was efficiently knocked down in the organoid model used in Figure 4E. It is therefore difficult to draw conclusions from this experiment.

- Some of the references do not match what is being stated. For example, Yap degradation in the cytoplasm references a commentary by Gumbiner and Kim, which does not discuss cytoplasmic degradation mechanisms for YAP. The authors should recheck their references, citing original data manuscripts if possible.

- The data shown in Fig 4 is interesting and suggests a biological significance of UBTD1 in human

cancers. It is however unclear whether this data supports the mechanisms proposed in the manuscript. For example, do UBTD1 levels anticorrelate with YAP target genes or beta-catenin target genes in tumor samples? Do the effects observed following UBTD1 knockdown on migration and invasion depend on YAP?

- It is unclear how many samples were analyzed in Figures 4G and 4H, and whether there is any statistical significance in these analyses?

Minor comment:

- The logic for why certain experiments were performed is not well justified. The manuscript reads as though the authors randomly tested associations between UBTD1 and mechanically responsive proteins. The authors should consider rearranging the presentation of the data.

Referee #3:

UBTD1 interacts with some of E2 and E3Ligases of the ubiquitin proteasome system. Considering its expression pattern in cells under mechanical stress and its down regulation in gastric cancer cells, this study explores the potential role of UBTD1 in mechanosensing. This manuscript demonstrates that UBTD1 is linked to the cellular contractile machinery. AFM and TFM experiments show that UBTD1 depletion induces a significant increase in the elastic modulus and in traction force demonstrating the involvement of UBTD1 in force generation. The localization of UBTD1 is sensitive to physical properties of extracellular matrix since UBTD1 re-localizes in cell-cell junction in stiff conditions. UBTD1 is associated with cadherin/catenin complex. UBTD1 deletion participates in YAP degradation through its association with the UbcH5c/b-TrCP complex and YAP complex.

Overall, the experiments reported in this study are well performed and controlled and data are relatively novel. However, at this stage the report lacks sufficient mechanistic insights in the link between UBTD1, cell-cell contact and YAP biology. The authors need to discriminate the role of UBTD1 at cell-cell contact and in the cytoplasm. Some issues that are listed below should be addressed in order to strengthen the manuscript.

General comments:
Two cell lines are used. It would be useful to indicate the type of cell lines in the text and on the different panel of each figure (For example, see fig 2F and fig. EV2 D).
YAP should be better introduced. Explanations about its interaction with b-catenin and UbcH5c/b-TrCP complex are missing. This would strengthen the rationale of the study.
Because YAP is sensitive to cell density, interpretation of data and final schema need to include this aspect. Images of cell density need to be shown in Fig. 2A and 3A. TFM need to be performed in isolated cells and cell monolayer (Fig. 1E). What about the localization of UBTD1 in isolated cells? The authors need to be precise: soft gel, stiff gel and plastic.

Major points:
PLA experiments: Controls have to be included in all PLA experiments. The suggestion would be to include the condition where UBTD1 is silenced (Fig. 1F, 3H). Immunofluorescence of cell-cell contact need to be combined with PLA experiments to see where the proximity ligation assay occurs.
Neither PLA experiment nor immunoprecipitation allow stipulating direct interaction between b-catenin and UBTD1. Replace "UBTD1 interacts with..." by "UBTD1 is found in the same complex as..." or "..is associated with b-catenin complex".
Some experiments are performed with fibronectin as ligand, some other one with Collagen. Why?
Fig. 1: Assuming that RhoA activity is measured in isolated cells (Fig1B), what is the activity of RhoA in cell monolayer? The distribution of focal adhesions needs to be analyzed in Fig. 1D to show the difference observed in fig. 1C but not commented.
Fig. EV1: b-catenin staining is missing in panel D, E and G. Treatment with Y27632 should be performed both in sparse and confluent cells. Treatment with Y27632 is not enough considering the

localization of UBTD1 in cell-cell contact. ROCK2 has been shown to be important in cell-cell contact. The authors would strengthen their study by showing the localization of UBTD1 in conditions where ROCK1 or ROCK2 is deleted to discriminate the activity of ROCK1 and ROCK2 on UBTD1.
Fig. 2 and 3: The authors claimed that UBTD1 is interacting with b catenin at cell-cell adhesion sites. There is no proof to demonstrate the direct interaction. Immunoprecipitation and PLA experiments show that UBTD1 belongs to the same complex. Biochemical studies with purified proteins would provide this proof. Moreover what is the role of UBTD1 at cell-cell contact? One issue would be to better understand the localization of UBTD1 at cell-cell contact. It would be important to investigate more in depth the involvement of UBTD1 in the cross-talk between YAP and b-catenin. According Azzolin et al (Cell 2014), YAP/TAZ are essential for b-TrCP recruitment and b-catenin inactivation and direct interaction between YAP and b-catenin has been already described (See Deng et al, cell death and disease, 2018, Azzolin et al 2012). The nuclear localization of YAP is an event that favors b-catenin nuclear localization by interfering with TRcP complex and b-catenin degradation. In this context, the condition without UBTD1 would be essential to show whether UBTD1 is important to keep b-catenin in UbcH5c/b-TrCP complex, to degrade b-catenin, to send b-catenin into nucleus and to activate b-catenine dependent transcription genes. What is the consequence of loss of cell-cell contact integrity (EGTA, HGF conditions) in YAP/b-catenin complex? Cellular fractionation would be useful in conditions where UBTD1 is deleted (fig. 2D).
Fig 2G and 2H are not clear to demonstrate b-catenin relocalization, quantification is needed.
The authors show that UBTD1 staining and cadherin staining are not overlapping (Fig. 2B). Does it mean that UBTD1 is more involved in actin organization? Considering the membrane localization of UBTD1 (Fig.2D) and its close proximity to b-catenin shown by PLA and IP (Fig.2E and 2F), could we expect co-sedimentation between actin and UBTD1 in cell monolayer?
Fig. 3A: quantification would be useful.
Fig.4E: better spheroid images should be provided.
Fig. EV4: what is the rationale to measure mRNA ROCK1 and ROCK2? Western blot and mRNA expression for ROCK1 and ROCK2 should be combined. Again the activity of ROCK1 and ROCK2 should be tested to analyze their specific effect on UBTD1 localization, YAP dependent genes, b-catenin-dependent genes etc..in sparse and confluent cells.

Minor points:
The term of spheroid is more appropriate than organoid.
Draw the limit of cells in TFM experiments.

1st Revision - authors' response 7 November 2018

Referee#1:

In this manuscript, the role of UBTD1 is examined and is shown to affect the mechanical properties of cancer cells, regulates YAP protein levels and promotes with tumorigenic properties in cells and is correlated with disease status in prostate and lung cancer patients. Activation of the Hippo pathway leads to LATS-mediated phosphorylation of YAP, which is followed by ubiquitination and degradation of Yap. Here, it is shown that siUBTD1 leads to an increase in Yap protein levels through the ability of UBTD1 to recruit beta-TrCP and the ubiquitination machinery to YAP. Assuming that the experiments can be reproduced with the appropriate controls (see below), the results showing that YAP levels increase upon loss of UBTD1 are convincing.
We are very grateful to the reviewer for this highly positive judgement.

1.However, the authors' conclusion that UBTD1 functions independent of Hippo, is not sufficiently supported by the very weak data provided (see specific comments below). This is not a key point of the paper, and either Hippo-dependent or independent function would be equally interesting, but if the authors wish to make the point of Hippo-independent, additional lines of experimental evidence are required to support this statement.
To address this important issue and to determine whether UBTD1 effect on YAP was

Hippo-dependent or independent, we took advantage of the cell density responsiveness of the Hippo pathway combined with specific knock-down of the kinase LATS1/2. Thus, we performed additional experiments at low and high density and using siLATS1/2 to inhibit Hippo pathway. New figures 3B-C and EV3A-B illustrate that UBTD1 depletion increased YAP protein level either at low and high cell density and did not modify LATS and MST phosphorylation. Moreover, new figures 5E-G and EV5E-G described the additive effect of Hippo inactivation by LATS knock-down and UBTD1 depletion on YAP nuclear translocation. Collectively this new set of data allows us to conclude that UBTD1 function on YAP protein level and nuclear translocation is Hippo independent.

2. The majority of the conclusions drawn rely heavily on the use of siUBTD1. Thus, it is absolutely essential that the knockdown efficiency be shown in every (or at least the majority of panels) in order to draw the conclusion that the observed effects are at least correlated with loss of expression of UBTB1 (as opposed to off-target effects). Disturbingly, knockdown efficiency by PCR is not shown anywhere in the manuscript (main or EV) and while a reduction in UBTD1 protein by immunoblotting was shown in 2 panels (Fig 1b and 3b), these data were for rather minor points. The authors have done qPCR and blots for many of the experiments reporting the effects of siUBTD1, thus there is no reason why the knockdown could not be shown in parallel. It would not be sufficient to add data showing the knockdown efficiency in a separate conducted experiment as part of a rebuttal, rather all of the key experiments would need to be repeated.
We agree with the reviewer and we have now included new experimental data with the appropriated controls to validate knockdown efficiency by qPCR, western blot or immunofluorescence in the main and EV figures.

3. It is known that siRNAs can have off-target effects, thus it is important to test key points with multiple siRNAs (ie deconvolve the pool used in the study).
Initially, to overcome result mis-interpretation due to SiRNA sequence off-target, we performed UBTD1 knock-down experiments by using a “siRNA pool” (4 different siRNA sequences) to dilute sequence-specific off-target effects. “SiRNA pool” also have the advantage of using lower individual siRNA concentration. Following the reviewer’s suggestion, we confirmed the effects of the UBTD1 “siRNA pool” by using, in most key experiments (in parallel), a unique siRNA sequence against UBTD1 (siRNA single1). New figures 1G, 4E, 5C-D, 6A, 6C-D and EV 1D, 5C-D, 6A, 6C-G show that “single siRNA” displays the same effect than the “siRNA pool” on UBTD1 depletion associated effects.

Rescue experiments with siResistant UBTD1 is also a standard requirement for siRNA experiments. Alternative knockdown approaches would also be valuable (CRISPR, shRNA) though not essential.
We particularly appreciated this reviewer suggestion and performed the “rescue experiment”. As proposed, we used a siRNA against a non-coding UBTD1 sequence (siResistant): “single2” in the manuscript, in cells transiently transfected with a human UBTD1 expressing plasmid (GFP-UBTD1). The result of the “Rescue experiment” nicely complements previous experiments and strengthen our conclusion showing that UBTD1regulates YAP protein level and nuclear translocation (Fig 5C-D and EV5C-D).

4. The authors should confirm the specificity of the UBTD1 antibody in IF experiments under both soft and firm ECM conditions (where changes in localization are observed) and in both cell lines using siRNAs.
In both cell lines, we have repeated the experiments (soft and firm ECM) in presence of siRNA against UBTD1 (siUBTD1 pool). New Figure 1H and EV1E, 2J include these control conditions.

5. Fig. 2f and 3H. PLA experiments. The authors should include at least one negative

control to confirm that the signal is specific (such as siUBTD1, and/or results when only one of the PLA protein detection reagents is present).
In agreement with the reviewer's comment, we added, in the main and EV figures, the PLA experiments using siUBTD1 as "negative control" (figures 2F, 4C, EV2F and EV3F).

6. Fig. 3A. The authors indicate that 'As expected, upon increased cell density, YAP level increased and YAP proteins were phosphorylated on ser127. In the blots shown, there actually is no increase in YAP protein levels in DU145. In some cell lines a decrease in YAP/TAZ protein levels are observed upon increased cell density due to phosphorylation/degradation.
We apologize for this inadequate interpretation of the Fig 3A. As pointed out by the reviewer, the total YAP level remains broadly unchanged while the Hippo-dependent phosphorylation of YAP (S127) increases proportionally to cell density.

7. Fig. 3B. The authors state that level of YAP phosphorylation (P-ser127YAP/total YAP ratio) remained unchanged and reference Fig 3B and Fig EV3A. While the blot does show there is no increase in the level of p-YAP upon siUBTD1 (rather it seems that there is less), but since total YAP increases, one would conclude that the relative levels of phosphorylated YAP to total YAP actually decrease. The authors should quantitate this to provide support for their conclusion, that contrasts what is evident in the blot. What cell density was used as compared to the densities examined in Fig. 3A? As recommended by the reviewer, we have now quantified the ratio of p-(ser127)YAP/total YAP (Figure 3B and EV3A). As mentioned in the revised manuscript "In UBTD1-depleted cells, we noticed that the YAP level increased significantly (x2) either in low or high cell density, resulting in a sharp decrease (25%) in the level of YAP phosphorylation (p-(S127)-YAP / total YAP ratio) (Fig 3B and Fig EV3A)". The reviewer is right, and we apologize for this mistake. In Figure 3B, "low density" is up to 30% confluence and "high density" is ranging between 80-100% confluence.

8. Fig. 3C. To conclude that LATS/MST phosphorylation is not altered by siUBTD1, a positive control to confirm phosphorylation of LATS and MST can be efficiently detected in these cell lines is required (ie could try low vs high density). Moreover, since the results are negative, it is essential to confirm loss of UBTD1 protein by blotting aliquots in parallel.
As mentioned above, we have followed the reviewer's suggestion and added new experiments at low and high cell density. New figures 3B-C and EV3A-C illustrate that UBTD1 depletion increased YAP protein level in either low and high cell density and did not modify LATS and MST phosphorylation.

It would be useful to indicate which phospho-sites are being monitored in Mst/Lats (the methods simply indicate a catalog number) so it would be helpful to add this information, preferably in the figure but minimally in the methods.
In the revised manuscript, we noted down the phosphorylated sites in the figure legends and in the "Materials and Methods" section.

9. The authors neglected to include the data they referred to showing that "less PLA between YAP and UBTD1 when UBTD1 is localized at the cell-cell contact sites instead of the cytoplasm." We have now included E-cadherin staining to label the cell-cell contact in the PLA experiments and we observed that PLA signals between YAP and UBTD1 are almost absent at the cell-cell contact (Figure 4C).

10. Fig. 3J. The knockdown efficiency of siUBTD1 must be shown (in this and all other knockdown experiments examining target gene expression).
We have performed new experiments with appropriated controls for knockdown efficiency

(qPCR, western blot or immunofluorescence) in the main and EV figures.

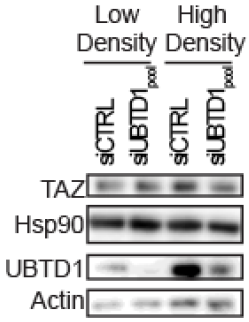
11. One conclusion of the data in Figure 3 is "that UBTD1 depletion is sufficient to induce a major upregulation of YAP signaling, independently of the Hippo pathway." Additional lines of experimental evidence are required to support this statement.
As mentioned above, we have done new experiments at low and high cell density in combination with siRNA against LATS1/2 to inhibit Hippo pathway. Thanks to the reviewer, we can now more firmly conclude that UBTD1 depletion is sufficient to induce a major upregulation of YAP signaling, independently of the Hippo pathway. In the revised manuscript, we also added a new set of data to show that UBTD1 effects on YAP signaling are ROCK2 dependent.

12. Fig. 4a: Does loss of YAP prevent siUBTD1-mediated EMT?
To answer this relevant question, we knock-down YAP/TAZ to determine whether the effects of UBTD1 depletion on EMT were YAP/TAZ dependent. As depicted on Figure 6A-B and EV6A-C, Knocking-down YAP/TAZ totally abrogates the effect of UBTD1 depletion on the expression of EMT markers and phenotypic features (migration and invasion) in both cell lines.

13. In Fig. 4, the authors show that siUBTD1 promotes various tumorigenic properties of cells. It would be important to show that the observed effects are dependent on YAP as opposed to a role for UBTD1 that is independent of YAP. A more balanced consideration of this possibility would enhance the study. As demonstrated by the new data added to the manuscript and in accordance with previous comment of
the reviewer (point n°12). We now show that acquisition of migrative and invasive properties of UBTD1 depleted cancer cells are under the control YAP/TAZ (Figure 6C-D and EV6F-G).

14. In the patient samples, is there a correlation between UBTD1 and Yap nuclear localization and/or target gene expression?
We are now able to provide a response to the reviewer since we show that, in patient samples, UBTD1 is correlated with BIRC5 a target gene of YAP (Figure EV6K).

15. Other points of curiosity: Does overexpression of UBTD1 decrease YAP expression and/or cause relocalization?
We now included in the manuscript GFP-UBTD1 over-expression experiments. As shown in Figure 5D and EV5D, UBTD1 over-expression decreases YAP nuclear translocation (Figure 5E and EV5C).



Does UBTD1 also act on WWTR1/TAZ? siCTRLsiCTRLHigh DensityLow DensitysiUBTD1poolsiUBTD1poolUBTD1ActinTAZHsp90
In DU145 and A549 cell lines, we did not observe any modification of TAZ protein level in UBTD1 depleted cells (*Figure R1)*.

Figure R1 : UBTD1 depletion has no effect on TAZ protein level

---
Referee #2:

The manuscript by Torrino et al. outlines a relationship between UBTD1 and mechanotranduction in the prostate and lung cancer cell lines. The authors report that UBTD1 localization is impacted by cytoskeletal changes induced by mechanical stimuli and that UBTD1 levels mediate cell tractions forces, RhoA activation, and YAP activity. The authors further report that prostate and lung cancer tissues show reduced UBTD1 levels, and that cancer patients with low expression of UBTD1 in their biopsies have poorer long-term survival.
UBTD1 is a ubiquitin-domain protein that has not been well studied, and this manuscript is one of few that investigates the biological roles of this protein. This manuscript therefore reports findings that are important and interesting. The relationship with cytoskeletal remodeling and mechanotransduction is of particular interest given the increasing interest in understanding these signals in the context of disease. We thank the reviewer for her/his positive appreciation of our findings.

However, many of the conclusions made about UBTD1 molecular function are preliminary and need further support before the manuscript is suitable for publication. In particular, the mechanisms linking
UBTD1 to YAP regulation are poorly supported by the presented data. Several comments are outlined below that need to be addressed.
With the support of reviewer comments, we provide, in the revised manuscript a more comprehensive data set to reinforce the experimental work and robustness of our conclusions.

Comments:
1/- Many conclusions about UBTD1 localization are made by IF microscopy using an UBTD1 antibody. The authors need to validate that the UBTD1 staining shown is specific. This should be very easy given how efficient the knockdown is following siRNA treatment of the cells.
As mentioned above in response to reviewer 1 (point n°4), we included in the main and EV figures, IF experiments using siRNA against UBTD1 as “negative control”.

2/- The data relating junctional localization of UBTD1 and YAP localization is confusing. Some data, such as the data in Fig 1F, suggest that junctional UBTD1 localization correlates with nuclear YAP localization. Other data, such as the data in Figure EV1E, shows that junctional UBTD1 localization correlates with conditions that promote cytoplasmic YAP localization. Further, the model shown in Fig 5 is confusing with respect to YAP localization and activity.
If YAP regulation remains central to the authors conclusions about UBTD1, the authors need to provide a clear relationship between the factors with more much more insight into the molecular mechanisms impacting YAP localization and activity.
Coherently with microscopic observations, we observed using sub-cellular fractionation, that UBTD1 is present in the membrane fraction re-inforcing our initial results showing that UBTD1 is associated with ß-catenin at the cell-cell adhesion site (Fig 2E and EV2D). Moreover, even more faintly in A549 cells (Fig EV2D) than in DU145 (Fig 2D), a proportion of UBTD1 resides the cytoplasm. To determine where UBTD1 is associated with YAP (you may notice in our experiment, as published by others, that a non-negligible part of YAP is present in the membrane fraction) we performed, as recommended by reviewers, PLA experiments between YAP and UBTD1 in E-cadherin stained cells to

delineate the cell-cell contacts (Fig 4C). Based on these new experiments, and mentioned less ambiguously in the revised manuscript, we can now claim that PLA interactions between YAP and UBTD1 occurred mainly in the cytoplasm.
To address the reviewer's concerns, we deciphered more in details the YAP-UBTD1 relationship, and for the sake of clarity, we separated the "YAP signaling" from the "YAP degradation" section in the revised manuscript. We now showed that UBTD1 is important for ß-TRCP-YAP association leading to YAP ubiquitylation and subsequent proteasomal degradation (Figure 4E-H and EV4D). Importantly, we provide some evidence demonstrating that the effect of UBTD1 on YAP localization (cytoplasmic/nuclear) and transcriptional activity is depending on ROCK2 (Figure 5I-K and EV5I-K).
According to additional experiments added to the manuscript, and reviewer's comments, we revised the graphical abstract to provide a more comprehensive and informative overview of our work.

3/- The reported increased levels of YAP with cell density that is shown in Fig 3A is surprising based on what has been reported about cell density/Hippo-mediated regulation of YAP. The authors should quantify the total levels of YAP in this experiment normalizing to an appropriate control. Also, by eye the levels of p-YAP shown in the western blot in Fig 3B appear to be reduced relative to the total YAP shown, and the total Yap may not be changing when taking into account the loading controls. The authors should quantify the p-YAP levels and recheck the quantitation of the total YAP normalized to the loading controls. If the authors do see a decrease in p-YAP, this should be reported in the manuscript. This issue has also been pointed out by reviewer 1: question n°6&7 and a detailed response to this concern is provided above. Briefly, we have now quantified the ratio of p-(ser127)YAP/total YAP (Figure 3B and EV3A). As mentioned in the revised manuscript "In UBTD1-depleted cells, we noticed that the YAP level increased significantly (x2) either in low or high cell density, resulting in a sharp decrease (25%) in the level of YAP phosphorylation (p-(S127)-YAP / total YAP ratio) (Fig 3B and Fig EV3A)".

4/- The authors make statements about UBTD1 impacting YAP independent of Hippo pathway signaling without actually testing whether any observed effects are indeed independent of Hippo pathway signaling. In fact, contrary to what is stated in the manuscript, the images presented in Fig 3B appear to show that phospho-YAP levels are changing upon UBTD1 knockdown, suggesting that the LATS-mediated phosphorylation of YAP is changing. The authors should recheck their quantitation and if Hippo-independent statements are to be made further experiments, experiments in the absence of LATS1/2 need to be performed.
We assumed that this point was insufficiently documented in the initial manuscript. We now provided a detailed set of experiments to clarify the involvement of the Hippo pathway on UBTD1 effects on YAP signaling and nuclear translocation. As previously documented in reply to reviewer 1 questions (7, 8 & 11), and following reviewer 2 recommendation, we performed experiments in both low and high cell density or using LATS 1/2 knock-down to specify the role of the Hippo pathway. In the revised manuscript, we provided converging evidence showing that UBTD1 depletion acts on YAP nuclear translocation and signaling in a ROCK2 dependent manner and independently of the Hippo pathway. Moreover, as recommended by the reviewers, we have now quantified the ratio of p-(ser127)YAP/total YAP (Figure 3B and EV re-inforcing 3A). As mentioned in the revised manuscript "In UBTD1-depleted cells, we noticed that the YAP level increased significantly (x2) either in low or high cell density, resulting in a sharp decrease (25%) in the level of YAP phosphorylation (p-(S127)-YAP / total YAP ratio) (Fig 3B and Fig EV3A)".

Please refer to additional response elements in the reply to reviewer 1.

5/- Given the effects on UBTD1 by cell density the authors need to state the confluence conditions that were used for each experiment.
Following the reviewer's comment, we now mentioned cell density conditions for each experiment.

6/- The conclusion in the manuscript related to YAP degradation being regulated by UBTD1 is very poorly supported by data. Given the central importance of this mechanism to the manuscript much more data showing an impact on YAP stability is needed. At a minimum, experiments that examine YAP protein turnover are required.
In agreement with this important comment of the reviewer, we have included a new data set in the revised manuscript to provide compelling evidence showing that UBTD1 depletion inhibits proteasomal degradation of YAP (Figures 3D-F and EV3C-D): To do so, we performed experiments using Cycloheximide (CHX) to inhibit protein synthesis or MG132 to inhibit proteasome degradation.

7/-If indeed YAP ubiquitination is regulated by UBTD1, it is unclear how the authors believe UBTD1 participates in YAP ubiquitination. Based on the data shown in Figs 3F and 3G UBTD1 and YAP stably interact, which is generally not the case for ubiquitin-regulating proteins that promote degradation.
As mentioned by the reviewer, most substrates did not interact stably with ubiquitin-regulating proteins. However, .as published previously by our collaborators, the GTPase Rac1 stably interacts with its ubiquitin ligase HACE1 leading to Rac1 proteasomal degradation (Torrino, et al. The E3 ubiquitin-ligase HACE1 catalyzes the ubiquitylation of active Rac1. Dev Cell. 2011 Nov 15;21(5):959-65. doi: 10.1016/j.devcel.2011.08.015). Similarly, it was also been demonstrated for YAP/TAZ with its ubiquitin ligase ß-TRCP (Azzolin, et al., YAP/TAZ incorporation in the β-catenin destruction complex orchestrates the Wnt response. Cell. 2014 Jul 3;158(1):157-70. doi: 10.1016/j.cell.2014.06.013).

More mechanistic insight into the roles of UBTD1 in YAP ubiquitination is required. How do the authors rationalize why a ubiquitin-like protein acts to promote polyubiquitin modification of YAP? If the authors believe that UBTD1 acts as an adaptor for beta-TRCP and/or UBCH5c, as drawn in the model in Fig 5, the authors should perform experiments to prove this. Alternatively, if the authors believe that the enzymatic activity of UBCH5 is affected by UBTD1, experiments to show such activity need to be included. To address the reviewer's concern, we performed immunoprecipitation of ß-TRCP in control or UBTD1 depleted cells. We found that UBTD1 depletion drastically inhibits YAP and ß-TRCP association leading to inhibition of YAP ubiquitylation and degradation (Figure 4G,H and EV4D). In the meantime, as shown in PLA experiments, UBTD1 depletion did not affect neither Ubch5c/ß-TRCP nor ß-catenin/ß-TRCP interactions suggesting a specific role at the level of YAP/ß-TRCP recognition confirmed by the
Co-IP result (Fig4E). Finally, the absence of PLA interaction between UBTD1 and ubiquitin excludes the possibility that UBTD1 is involved in ubiquitin transfer between UbcH5 and YAP (Fig EV4B).
Further technical arguments are discussed below (question n°11)

8/- The PLA data is not very convincing and is lacking several controls. No antibody controls are shown and often very few spots are detected. Ideally, each of the PLA experiments should be performed following UBTD1 knockdown to show that the observed signals are indeed representing interactions. In agreement with the reviewer, we added appropriated PLA control: siUBTD1 (figures 2F, 4C and EV2F) in both the main and EV figures.

9/- The experiment in Fig 3E does not properly address whether UBTD1 regulates YAP

ubiquitination, and the conclusions made by the authors are not supported by the presented data. First, the data as presented cannot exclude differences in the transfection of the His-Ub in the different samples.
We added the control of His-Ub transfection (Figure 3K; total: his). We did not see any difference in the transfection of His-Ub between the 2 conditions of interest (His-Ub+YAP and His-Ub+YAP+siUBTD1).

Also, often experiments examining direct modification of a protein by Ub in cells include a second IP to ensure the specificity of the protein being detected.
As mentioned it the "Material and Methods" section, we used an Urea buffer that disrupt no-covalent interaction while preserving covalent links (YAP-Ub interactions). This protocol is widely used and our collaborator, E. Lemichez published significant results by using this technique (Torrino *et al*, "The E3 ubiquitin-ligase HACE1 catalyzes the ubiquitylation of active Rac1" Dev cell 2011).

11/ If the authors propose that UBTD1 is part of the YAP ubiquitination complex, this experiment should be performed in vitro with purified components to prove this role for UBTD1.
As suggested by the reviewer, *in vitro* experiments with purified components will be an elegant way to undoubtedly demonstrate the role of UBTD1, but this is technically highly challenging. Indeed, to be performed, this experiment required not only purification of UBTD1, YAP, ß-TRCP and Ubchc5 but also all the proteins of the RING E3 ligase complex (such as Skp1, Culline, F-box). Thus, this is a very complex experiment that can't be done in a limited period of time. To overcome this problem (only a limited number of dedicated labs around the world are able to perform this kind of experiments) we performed immunoprecipitation of ß-TRCP in control or UBTD1 depleted cells. By this easier technical way, we found that UBTD1 depletion inhibits YAP and ß-TRCP interaction leading to inhibition of YAP ubiquitylation and degradation (Figure 4H). We further confirmed this result testing the various interactions between protein of the degradation complex by PLA experiment in UBTD1 depleted cells
(Figure 4F,G and EV4D). We believe that our results provide some creditable and consistence evidence on the role of UBTD1 in the YAP degradation complex.

12/- It is unclear why the total YAP levels in Fig 3E are not increased with UBTD1 knockdown. This data is inconsistent with the ubiquitin levels shown in the same figure and the conclusions made by the authors from prior figures.
Indeed, for this experiment, we overexpressed YAP to perform ubiquitylation assay and to obtain the same amount of YAP level. For this reason, YAP level in Fig 3E is not increased after UBTD1 knockdown.

13/- There is no validation that UBTD1 was efficiently knocked down in the organoid model used in Figure 4E. It is therefore difficult to draw conclusions from this experiment.
In full agreement with the reviewer's comment, we added in the new figure the qPCR knock-down efficiency (Figure EV6H).

14/- Some of the references do not match what is being stated. For example, Yap degradation in the cytoplasm references a commentary by Gumbiner and Kim, which does not discuss cytoplasmic degradation mechanisms for YAP. The authors should recheck their references, citing original data manuscripts if possible.
We apologize for this mistake, we corrected citations in the revised manuscript.

15/- The data shown in Fig 4 is interesting and suggests a biological significance of UBTD1 in human cancers. It is however unclear whether this data supports the mechanisms proposed in the manuscript. For example, do UBTD1 levels anticorrelate with

YAP target genes or beta-catenin target genes in tumor samples?
As it was also suggested by reviewer 1, we have analyzed the correlation between UBTD1 and YAP target genes or β-catenin target genes in tumor samples. We observed in patient samples that there is a correlation between UBTD1 and BIRC5 a target gene of YAP (figure EV6K). In accordance with the lack of effect of UBTD1 depletion on Wnt signaling in our cell models, we did not observe any correlation between β-catenin target genes and UBTD1 in patient samples. However, as extensively described in the manuscript, we showed that UBTD1 acts on YAP signaling through ROCK2. Accordingly, we observed an inverse correlation between UBTD1 and ROCK2 expression in patients' samples.

16/Do the effects observed following UBTD1 knockdown on migration and invasion depend on YAP? To determine the involvement of YAP on migration and invasion induced by UBTD1 depletion, we
knock-down YAP/TAZ. We found that loss of YAP/TAZ prevents both migration and invasion in UBTD1 depleted cells (Figure 6C-D and EV6F-G).

17/- It is unclear how many samples were analyzed in Figures 4G and 4H, and whether there is any statistical significance in these analyses?
For this analyze, we used 20 patients 'samples, this is now mentioned the new version of the manuscript. For prostate sample, we observed UBTD1 staining in whole normal prostate gland and no staining in all tumor cells. For lung sample, we observed UBTD1 staining in all alveolar normal cells and no staining in all tumor tissues and metastasis cells.

Minor comment:
- The logic for why certain experiments were performed is not well justified. The manuscript reads as though the authors randomly tested associations between UBTD1 and mechanically responsive proteins. The authors should consider rearranging the presentation of the data.
This has been done. We hope that the new manuscript organization will facilitate the reading and will underline the overall consistency of our work.

Referee #3:

UBTD1 interacts with some of E2 and E3Ligases of the ubiquitin proteasome system. Considering its expression pattern in cells under mechanical stress and its down regulation in gastric cancer cells, this study explores the potential role of UBTD1 in mechanosensing. This manuscript demonstrates that UBTD1 is linked to the cellular contractile machinery. AFM and TFM experiments show that UBTD1 depletion induces a significant increase in the elastic modulus and in traction force demonstrating the involvement of UBTD1 in force generation. The localization of UBTD1 is sensitive to physical properties of extracellular matrix since UBTD1 re-localizes in cell-cell junction in stiff conditions. UBTD1 is associated with cadherin/catenin complex. UBTD1 deletion participates in YAP degradation through its association with the UbcH5c/b-TrCP complex and YAP complex.

Overall, the experiments reported in this study are well performed and controlled and data are relatively novel. However, at this stage the report lacks sufficient mechanistic insights in the link between UBTD1, cell-cell contact and YAP biology. The authors need to discriminate the role of UBTD1 at cell-cell contact and in the cytoplasm. Some issues that are listed below should be addressed in order to strengthen the manuscript.
We are very grateful to the reviewer 3 for this highly positive judgement. We have done our best to keep the quality of this study during the revision process.
Following reviewer guidelines, we hope that the revised manuscript meets the reviewers

'expectations and strengthen our conclusions.

General comments:

1/Two cell lines are used. It would be useful to indicate the type of cell lines in the text and on the different panel of each figure (For example, see fig 2F and fig. EV2 D).
We now have indicated the cell line in each figure.

2/YAP should be better introduced. Explanations about its interaction with b-catenin and UbcH5c/b-TrCP complex are missing. This would strengthen the rationale of the study.
We modified the introduction of the manuscript according to the reviewer's comment.

3/Because YAP is sensitive to cell density, interpretation of data and final schema need to include this aspect. Images of cell density need to be shown in Fig. 2A and 3A.
We reorganized the manuscript, added several data, modified the final schema and included images of cell density according to the reviewer's comment (Fig EV2A).

4/TFM need to be performed in isolated cells and cell monolayer (Fig. 1E).
We followed the suggestion of the reviewer and we have now performed TFM experiments in cell monolayer (Figure 1G and figure EV1C) and isolated cells (Figure 1E,F and figure EV1D).

5/What about the localization of UBTD1 in isolated cells?
As shown in Figure EV1F and EV2K, UBTD1 is localized in the cytoplasm of isolated cells.
6/The authors need to be precise: soft gel, stiff gel and plastic.
We agree with the reviewer and we have now precise soft, stiff gel, glass or plastic.

Major points:

7/PLA experiments: Controls have to be included in all PLA experiments. The suggestion would be to include the condition where UBTD1 is silenced (Fig. 1F, 3H).
In agreement with the reviewers, we added appropriated PLA control: siUBTD1 (figures 2F, 4C and EV2F) in both the main and EV figures.

8/Immunofluorescence of cell-cell contact need to be combined with PLA experiments to see where the proximity ligation assay occurs.
We have now included E-cadherin staining to label the cell-cell contact in the PLA experiments.

9/Neither PLA experiment nor immunoprecipitation allow stipulating direct interaction between b-catenin and UBTD1. Replace "UBTD1 interacts with..." by "UBTD1 is found in the same complex as..." or "..is associated with b-catenin complex".
We are sorry for the mistake and misinterpretation in the initial manuscript. Furthermore, based on this reviewer remark, we provide, in the revised manuscript, new evidences to more precisely decipher how UBTD1 regulates YAP degradation in a dedicated section: "UBTD1 regulates YAP ubiquitylation by modulating its association with ß-TRCP".

10/Some experiments are performed with fibronectin as ligand, some other one with Collagen. Why?
For epithelial cells, both can be used and we observed the same effect on UBTD1 localization whatever the matrix. However, for sake of experimental homogeneity and as recommended by the reviewer, fibronectin alone was used for additional experiments included in the revised manuscript.

11/Fig. 1: Assuming that RhoA activity is measured in isolated cells (Fig1B), what is the activity of RhoA in cell monolayer?
In the original manuscript, our explanation was probably confusing. Indeed, we did this experiment in cell monolayer and observed that UBTD1 depletion increased RhoA activity.

12/The distribution of focal adhesions needs to be analyzed in Fig. 1D to show the difference observed in fig. 1C but not commented. A0501001502002500246810 12number of FAsiUBTD1poolsiCTRLDistance from Cell plasma (A.U.)
Distribution of focal adhesions has been analyzed and results are provided in Figure R2: UBTD1 depletion did not modify the distribution of focal adhesions.

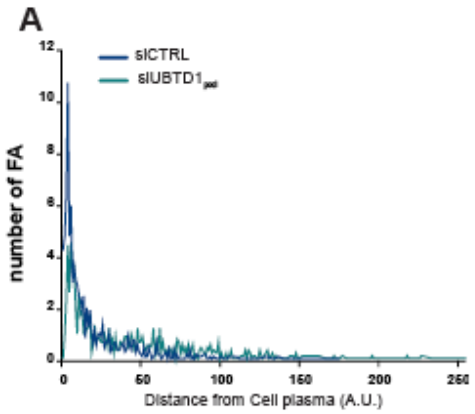


Figure R2: UBTD1 depletion did not modify the distribution of focal adhesion in DU145 cells.

13/Fig. EV1: b-catenin staining is missing in panel D, E and G.
We have now included figures EV 2J-L to show ß-catenin localization on soft or stiff gel, in sparse or confluent cells and upon cytoD treatment in control cells or UBTD1 depleted cells. UBTD1 depletion did not modify ß-catenin localization.

14/Treatment with Y27632 should be performed both in sparse and confluent cells.
We have now performed Y27632 treatment in sparse cells and observed that it did not modify UBTD1 localization in sparse cells (Figure R3). UBTD1CTRLY-27632

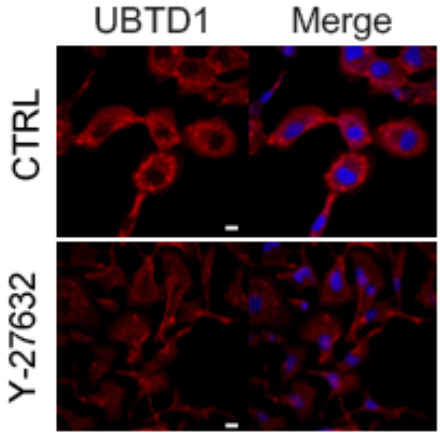


Figure R3: Representative confocal images showing UBTD1 localization after Y-27632 in isolated DU145 cells.

15/Treatment with Y27632 is not enough considering the localization of UBTD1 in cell-cell contact. ROCK2 has been shown to be important in cell-cell contact. The authors would strengthen their study by showing the localization of UBTD1 in conditions where

ROCK1 or ROCK2 is deleted to discriminate the activity of ROCK1 and ROCK2 on UBTD1.
This is indeed an important question. We have therefore performed immunofluorescence in cells depleted for ROCK1 or ROCK2 and we did not observe any modification of UBTD1 localization (Figure 5I and EV 5I).

16/Fig. 2 and 3: The authors claimed that UBTD1 is interacting with b catenin at cell-cell adhesion sites. There is no proof to demonstrate the direct interaction. Immunoprecipitation and PLA experiments show that UBTD1 belongs to the same complex. Biochemical studies with purified proteins would provide this proof.
We totally agree with the reviewer and according to her/his previous comment (n°9), we gave a more careful interpretation of our result in the revised manuscript.

17/Moreover what is the role of UBTD1 at cell-cell contact? One issue would be to better understand the localization of UBTD1 at cell-cell contact.
This is clearly an important issue that require to be addressed independently since it exceeds the frame of the present manuscript. However, considering the crosstalk between YAP/TAZ and the Wnt signaling pathway, and to give some the reader partial response element about this topic, we performed some experiments to evaluate a potential role of UBTD1 in the Wnt pathway. In our cell model, we have not been able to see any effect of UBTD1 on Wnt signaling. But definitely, the role of UBTD1 at the cell adhesion is a crucial point that we will address in the near future.

18/It would be important to investigate more in depth the involvement of UBTD1 in the cross-talk between YAP and b-catenin. According Azzolin et al (Cell 2014), YAP/TAZ are essential for b-TrCP recruitment and b-catenin inactivation and direct interaction between YAP and b-catenin has been already described (See Deng et al, cell death and disease, 2018, Azzolin et al 2012). The nuclear localization of YAP is an event that favors b-catenin nuclear localization by interfering with TRcP complex and b-catenin degradation. In this context, the condition without UBTD1 would be essential to show whether UBTD1 is important to keep b-catenin in UbcH5c/b-TrCP complex, to degrade b-catenin, to send b-catenin into nucleus and to activate b-catenin dependent transcription genes.
We followed the suggestion of the reviewer and we have performed immunoprecipitation of ß-TRCP in UBTD1 depleted cells. Interestingly, UBTD1 depletion did not alter the interaction between UbcH5c and ß-TrCP and did not perturb ß-catenin in UbcH5c/b-TrCP complex (Figure 4E). Moreover, UBTD1 depletion did not modify the localization of ß-catenin (figure EV2) and had no effect on ß-catenin target genes (Figure R4). AB01234 Nuclear / Cytosolic b-catenin (A.U.) Cnsns050100150mRNA expression (% of siCTRL)050100150200Axin2c-MYCUBTD1Axin2c-MYCUBTD1****mRNA expression (% of siCTRL)DU145 A549siCTRLsiUBTD1poolsiCTRLsiUBTD1poolsiCTRLsiUBTD1poolsiCTRLsiUBTD1poolDU145 A549b-cateninsiCTRLMergesiUBTD1poolb-cateninMergeDU145 A549

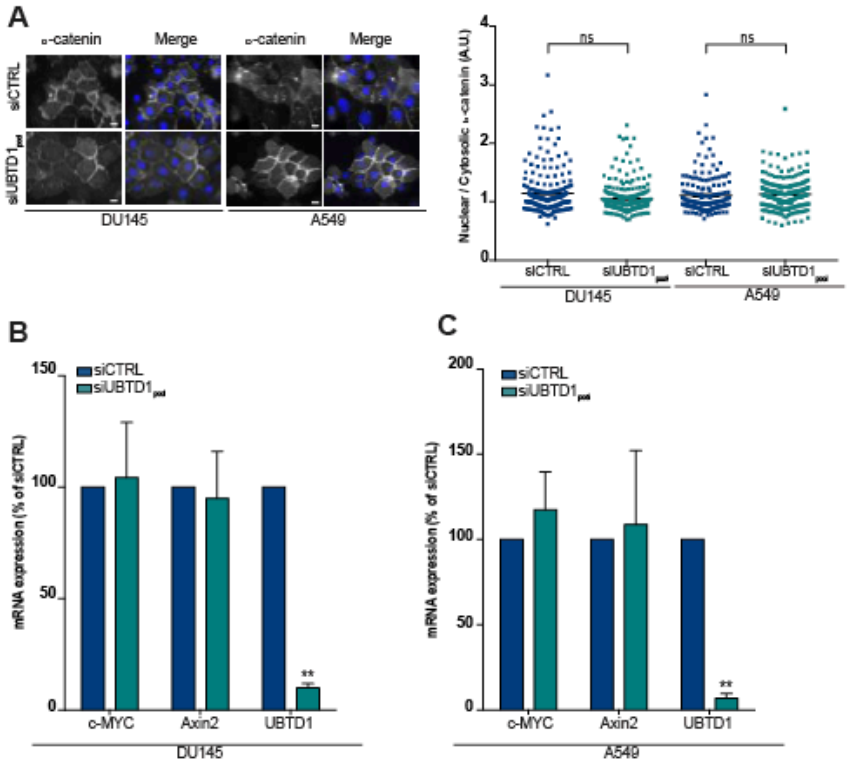


Figure R4 : UBTD1 did not affect ß-catenin localization and had no effect on ß-catenin target genes.
(A-C) DU145 or A549 cells were transfected with the indicated siRNA (control, siCTRL; UBTD1, siUBTD1pool). (A) Representative wide-field immunofluorescence images (left) and quantification (right) showing endogenous ß-catenin localization. (B-C) Quantification of c-MYC, Axin2 and UBTD1 mRNA levels.

19/What is the consequence of loss of cell-cell contact integrity (EGTA, HGF conditions) in YAP/b-catenin complex? Cellular fractionation would be useful in conditions where UBTD1 is deleted (fig. 2D).
To address the reviewer's concern, we performed cellular fractionation in UBTD1 depleted cells. UBTD1 depletion increased YAP level and its nuclear localization (Figure 2D and EV 2D). However, UBTD1 depletion did not modulate neither ß-catenin level nor its localization and did not trigger a Wnt dependent transcriptional response (Figure R4). Given the absence of UBTD1 effect on ß-catenin localization and expression, we did not further explore this peculiar point.

20/Fig 2G and 2H are not clear to demonstrate b-catenin relocalization, quantification is needed.
As shown in Figure R4A, UBTD1 depletion did not alter either nuclear translocation of ß-catenin or expression of target genes. Because of this negative result, we mentioned this important point in the revised manuscript, but we didn't include Figure R4 to the supplementary data section.

21/The authors show that UBTD1 staining and cadherin staining are not overlapping (Fig. 2B). Does it mean that UBTD1 is more involved in actin organization? Considering the membrane localization of UBTD1 (Fig.2D) and its close proximity to b-catenin shown by PLA and IP (Fig.2E and 2F), could we expect co-sedimentation between actin and UBTD1 in cell monolayer?
This is a very interesting remark. As described in the revised version of the manuscript, even in the absence of cell contact (low cell density), UBTD1 depletion drastically

modifies acto-myosin contractility, activates RhoA (Fig 1) but also increases YAP level (Fig 3B). In addition, we now show that UBTD1 acts on YAP signaling through ROCK2 suggesting an inhibitory function of UBTD1 on RhoA/ROCK2 axis. Since the RhoA/ROCK signaling platform is controlled by the cytoskeleton dynamics, a role of UBTD1 on this system is highly probable. In addition, as observed on Figure R5, UBTD1 does not clearly overlap with actin cytoskeleton in sparse cells (even if it localizes sometimes nearby), suggesting that UBTD1 may be involved in the functionality of the actin network but not associated with actin "by itself". This argumentation is further supported by an undisclosed result of a proteomic analysis that we have done, and we would like to publish as a "full story" in the next future. We discussed extensively this aspect in the discussion section of the manuscript. We hope that the reviewer will understand this point of view and will be convinced by our additional data enclosed in the revised manuscript.

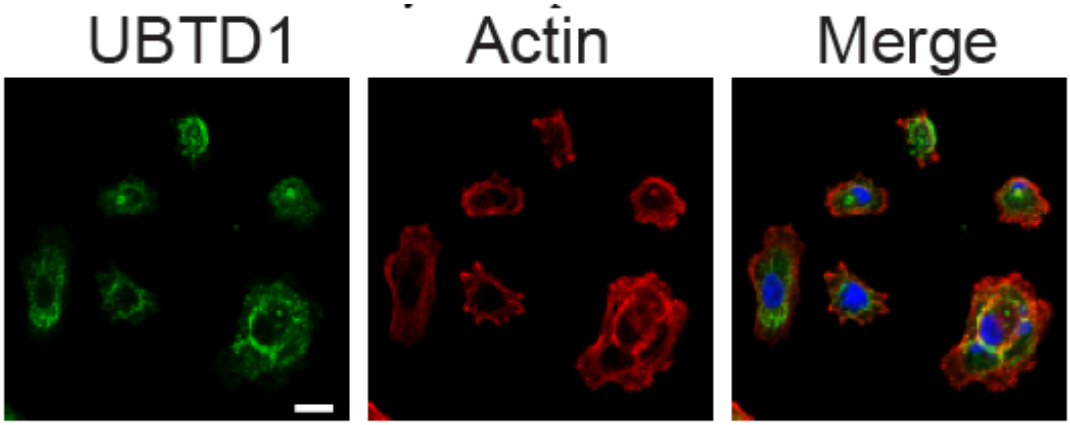


Figure R5 : *UBTD1 does not overlap with actin network in sparse DU145 cells*

22/Fig. 3A: quantification would be useful.
To complement Fig 3A, we provided an additional experiment to determine the effect of cell density on the increase of YAP level induced by UBTD1 depletion (Fig3 B). Quantifications of the total YAP and p-YAP/YAP are illustrated by bar graphs in Fig 3B and discussed in the revised manuscript.

23/Fig.4E: better spheroid images should be provided.
We changed the image for the UBTD1 depleted condition, but due to transmission microscopy and cell compaction degree within human tumoroids (much harder to manipulate than classical cell line spheroids), illustration is not as nice as we would like.

24/Fig. EV4: what is the rationale to measure mRNA ROCK1 and ROCK2? Western blot and mRNA expression for ROCK1 and ROCK2 should be combined. Again the activity of ROCK1 and ROCK2 should be tested to analyze their specific effect on UBTD1 localization, YAP dependent genes, b-catenin-dependent genes etc..in sparse and confluent cells.
We thank reviewer 3 for her/his important remarks related to the ROCK. Following her/his recommendations, we performed a new set of experiments and, importantly, we found that the effect of UBTD1 depletion on YAP signaling (nuclear translocation and transcriptional activity) was ROCK2 dependent. New figures 5H-K and EV 5H-K showed that ROCK1 or ROCK2 depletion did not affect YAP expression and as mentioned above, ROCK1 and ROCK 2 depletion did not affect
UBTD1 localization. We extensively modified the revised manuscript and we adapted the summary schema according to this important finding.
As explained above (question 18), UBTD1 depletion did not modify either ß-catenin nuclear translocation or Wnt transcriptional activity (Figure R4). Moreover, neither

ROCK1, ROCK2 or UBTD1 depletion affect ß-catenin protein level suggesting no/limited impact on Wnt signalling (Figure R6).

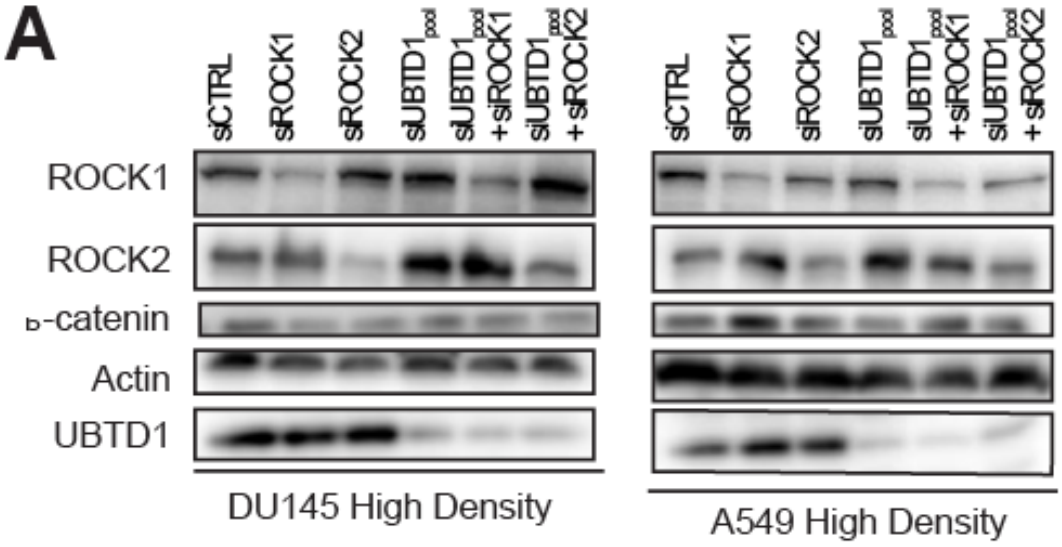


Figure R6: ROCK1, ROCK2 or UBTD1 depletion did to affect ß -catenin protein level in DU145 or A549 cells.

Minor points:

The term of spheroid is more appropriate than organoid.
In the revised manuscript we use the term "human tumoroid" as recommended by Dutta, D. and Clevers H. ("Disease Modeling in Stem Cell-Derived 3D Organoid Systems". Trends Mol Med. 2017;23(5):393-410). Indeed, the term "spheroid" is referred to a 3D cancer cell line culture whereas "tumoroid" designate cancer cells directly collected from a patient tumor and cultivated *ex-vivo* which required a protocol adapted from "organoid culture" and is much more challenging (at least for prostate cancer).

Draw the limit of cells in TFM experiments.
This has been done in the revised version of the manuscript.

2nd Editorial Decision 17 December 2018

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your manuscript in EMBO reports. However, referees #2 has some remaining concerns or further suggestions we ask you to address in a final revised version of your manuscript.

Further, I have these editorial requests:

- Please add the chapter names 'Introduction' and 'Results' to the manuscript text.

- Please add a running title (not more than 40 characters including spaces), and up to five key words to the title page.

- Please add a conflict of interest statement to the manuscript text (below the author contributions).

- It seems that authors Sabrina Pisano (or is she P.S.?), Jay P. Uhler, Emmanuel Lemichez, Amel Mettouchi and Christophe Lamaze are not mentioned in the author contributions. However, and author P.S. is mentioned, that is not on the title page (or should that be Sabrina Pisano?). Please correct this, and make sure that each author of this paper is mentioned with appropriate contribution

here.

- Author C. Gaggiolo is mentioned as 'supervised the core facility'. Are you sure that this author contributed substantially for the manuscript, following the ICMJE guidelines for authorship. Or would a mention in the acknowledgements be sufficient? See:
http://www.icmje.org/recommendations/browse/roles-and-responsibilities/defining-the-role-of-authors-and-contributors.html

- Per journal policy, we do not allow 'data not shown' (see page 8 and 17 of your manuscript). All data referred to in the paper should be displayed in the main or Expanded View figures, or the Appendix. Thus, please add these data (or change the text accordingly, if these data are not important). See:
http://embor.embopress.org/authorguide#unpublisheddata

- Presently, Fig 2F is called out before Fig 2D. Please change this, or the order of the panels in the figure.

- Many of the immunofluorescence images are not showing all colours in the merge, or show new colours in the merge. In many cases there are now blue colours in the merge that do not show up in one of the other panels. E.g. in panel 1H, there is no signal in UBTBD1 upon KD, but in the merge we see large blue regions. The same in the siRNA control, though here there is additional UBTBD1 staining in red. Most of the merged images show these blue signals. Please explain.
- There are some immunofluorescence images, which show a merged image, but there is only one further image. (e.g. 5A, EV1G/I or EV4A). What was merged here? Please explain.

- As I mentioned in my previous letter, up to 5 images can be displayed as Expanded View. You have presently uploaded 6 EV figures, and some of these extend over 2 pages. We cannot proceed with these. Please provide 5 EV figures, each on one page. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section.

See also our guide for figure preparation:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

- The remaining supplementary material should be supplied as a single pdf labeled Appendix (see below). Please add page numbers to the Appendix, and a TOC (table of contents) with page numbers, and name the file 'Appendix'. Please use the nomenclature Appendix Figure Sx or Appendix Table Sx for Appendix items, and change the callouts accordingly throughout the manuscript text.

- There are tables in the methods section. Either name these as Tables and call these out accordingly in the text, or move these to the Appendix (with the nomenclature Appendix Table Sx).

- Please provide scale bars for Fig. 6G/H and EV6J. Please check carefully that all microscopic panels have scale bars.

- Panels in Fig EV3A and EV3B are partly duplicated. Please explain, and/or add an explanation to the figure legend.

- As they are significantly cropped, please provide the source data for the entire Western blots shown in the manuscript (including the EV figures and the Appendix figures)? The source data will be published in separate source data files online along with the accepted manuscript and will be linked to the relevant figures. Please submit scans of entire gels or blots together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

- Regarding data quantification and statistics, please check for all diagrams that the number "n" for how many independent experiments (biological replicates) were performed and also the test used to calculate p-values is indicated in the respective figure legends. See:

http://embor.embopress.org/authorguide#statisticalanalysis

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

- Please provide an ORCID for both corresponding authors, and link these to their EMBO reports author accounts.

When submitting your revised manuscript, we will require:
- a Microsoft Word file (.doc) of the final revised manuscript text
- a point-by-point response addressing the final referee concerns
- editable TIFF or EPS-formatted figure files (main figures and EV figures) in high resolution (of those with adjusted panels or labels).
- The Appendix file.

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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

REFEREE REPORTS

Referee #1:

The authors have adequately addressed all of my concerns.

Referee #2:

The revised manuscript has addressed the majority of the comments brought up in the initial review and overall the manuscript describes interesting and novel data. However, there are some comments I have related to the new data that the authors should try and address before publication of the manuscript.

- The data shown for the different siRNA treated samples in Figure 5F and 5I appear to be from cells at vastly different confluence. It is therefore very difficult to draw comparative conclusions from this data.

- The argument that UBTD1 functions independent of LATS1/2 (Hippo) signaling is still weak, as the experiments testing LATS1/2 independence do not show a complete loss of LATS protein (not sure whether the data in 5F shows LATS1 or LATS2?). My suggestion is for the authors to reword the text in the manuscript to lessen the argument that UBTD1 acts independent of LATS signaling.

- The quantitation in figure 3F is unclear. Are the numbers relative to the 0h siCTL? Do the siCTL and siUBTD1 samples show the same levels of YAP? If not, then why are YAP levels not increased in the A549 cells?

- The n for the data in Figure 6F should be included.

Referee #3:

The authors elucidate the biological function of UBDT1 in mechanosensing and cancer through its

implication in YAP degradation complex and in control of YAP signaling via ROCK2 (in an independent manner of Hippo pathway). First the results show that UBTD1 is linked to the contractile machinery: (i) UBDT1 expression is regulated by cell-cell contacts, (ii) UBDT1 is associated with b-catenin in cell-cell contacts and (iii) UBDT1 accumulates in cell-cell contact when the matrix is stiff and is delocalized after actin network disruption. Second, UBDT1 is crucial for limiting force generation. Third UBDT1 is associated with components of YAP degradation complex by promoting YAP-TrcP interactions and ensuring efficient YAP ubiquitinylation. UBDT1 depletion decreases YAP ubiquitylation and triggers ROCK2 dependent YAP signaling activation. Finally the authors show that UBDT1 represses cancer aggressiveness by impacting EMT markers,migration and invasion processes. Considering UBDT1 expression in cells experiencing mechanical stress, this mechanism provides first evidence to explain the correlation between patient survival and UBDT1down regulation in gastric cancer cell lines and tissue. The manuscript has been greatly improved in term of data and clarity. The manuscript is now acceptable for publication in EMBO reports.

2nd Revision - authors' response 21 December 2018

Response to Referee #2

The revised manuscript has addressed the majority of the comments brought up in the initial review and overall the manuscript describes interesting and novel data. However, there are some comments I have related to the new data that the authors should try and address before publication of the manuscript.
We thank the reviewer for his/her final comments and recommendations.

- The data shown for the different siRNA treated samples in Figure 5F and 5I appear to be from cells at vastly different confluence. It is therefore very difficult to draw comparative conclusions from this data.
As well documented, ROCK knock-down (particularly ROCK1 in our cell lines) reduces cell adhesion. Inevitably, there is some slight differences in cell densities (representative images in Fig 5I). Unlike ROCK1/2 knock-down we do not notice any change in cell confluence after LATS1/2 knock-down. Therefore, we adjust cell seeding (same number) just to reach confluence 48h later. Consequently, even if the cells are broadly confluent in the well, some slight variations persist. Then we proceed to random imaging (hundreds of cells are analyzed) within each well to correct these differences. Data analysis is represented on the scatter plots graph.

- The argument that UBTD1 functions independent of LATS1/2 (Hippo) signaling is still weak, as the experiments testing LATS1/2 independence do not show a complete loss of LATS protein (not sure whether the data in 5F shows LATS1 or LATS2?).
We knock-down both LATS1 and LATS2. LATS1 level (cell signaling 9153) was checked by western-blot. Figures 5E et EV4E were corrected accordingly.

My suggestion is for the authors to reword the text in the manuscript to lessen the argument that UBTD1 acts independent of LATS signaling.
Modified in the manuscript as recommended by the reviewer.

- The quantitation in figure 3F is unclear. Are the numbers relative to the 0h siCTL?
The quantifications represent the ratio between 6h treatment/0h for each SiRNA condition (CTRL or UBTD1)

Do the siCTL and siUBTD1 samples show the same levels of YAP? If not, then why are YAP levels not increased in the A549 cells?
There is a slight (compared to DU145) increase in YAP level in SiRNA UBTD1 condition compared to SiRNA control. SiUBTD1/SiCTRL ratio at t=0 is 1.2

- The n for the data in Figure 6F should be included.
Modified in the figure legend as recommended by the reviewer.
Normal: patient number n=47

Tumor: patient number n=70

3rd Editorial Decision 11 January 2019

I now went through the manuscript, and I think that the remaining concerns of referee #2 have been adequately addressed. However, some editorial issues remain.

I would suggest a slightly different title:
Ubiquitin domain-containing protein 1 is a mechano-regulator controlling cancer aggressiveness

Please mention in the respective figure legends that panels in Fig EV3A and EV3B are partly duplicated. It seems also in Fig. 3B/C some panels show up twice. Please mention all these cases in the respective figure legends.

Please add scale bars to Figs. 6J and EV1A.

Could in Fig. 4A/B the similar areas of the blots be shown for A549 and DU145 (same size)?

The source data (SD) is presently very confusing. We would require the following changes:

- Please provide one source data file per figure, containing only data referring to that figure. Presently, some source data files for main figures do also contain panels referring to EV figures, and some source data files for EV figures do not contain all the relevant panels. Each source data file (for main and EV figures) will be linked to the respective figure, and should therefore contain all the relevant data, but should also only contain data shown in the respective figure.

- Please group the panels in the source data files in the order they are shown in the figure, and give them the same call outs. It is confusing if e.g. panels labelled as B in SD file for Fig. 2 show data for panel 2E.

- Please show the panels in the source data with the same intensity/contrast/exposure as in the manuscript Figure. Presently, in some cases these are very different, and sometimes it is hard to see that the panels show the same data.

- There are several panels where the source data does not match to the figure panels (e.g. EGFR in Fig2E - there are many more, I gave up comparing while looking at the SD for Figure 4). Please carefully revise this, go through all the SD, and make sure that all the SD panels show indeed the same blots and exposures as shown in the final figures!

Finally, EV Figures 2-5 are extremely crowded, and some of the smaller panels won't show very well in the final online version of the paper (e.g. panels E, F and H in EV3). Could you please re-assemble these figures, remove some data, and move these to one or two Figures that will be included into the Appendix as Appendix Figure Sx? Please then also update all the call-outs in the manuscript text accordingly. See also our guide for figure preparation:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

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

3rd Revision - authors' response 18 January 2019

The authors performed all minor editorial changes.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Clavel
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-46570V2

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

USEFUL LINKS FOR COMPLETING THIS FORM

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

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

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

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical method to predetermine sample size was used. Experiments are independently repeated at least 3 times.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data was excluded
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NO
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes the data meets the assumptions of the test
Is there an estimate of variation within each group of data?	reported as SEM as indicated
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The companies and catalogu numbers for antibodies used in the study were described in the method section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	DU145, A549 and HEK were purchased from the American Type Culture Collection (ATCC)

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	CPP Sud Mediteranee V
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	See attached file "patient consents"
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No