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HERC3 promotes YAP/TAZ stability and tumorigenesis independently of its ubiquitin ligase activity

Bo Yuan, Jinquan Liu, Aiping Shi, Jin Cao, Yi Yu, Yezhang Zhu, Chengbin Zhang, Yifei Qiu, Hongjie Luo, Jiaxian Shi, Xiaolei Cao, Pinglong Xu, Li Shen, Tingbo Liang, Bin Zhao, and Xin-Hua Feng
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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received comments from three reviewers, which are included below for your information.

As you will see from the comments, all reviewers appreciate the study. However, they also find that further reaching experiments are needed to clarify a number of important aspects of the proposed HERC3-mediated regulation of YAP via inhibition of beta-TRCP activity, including further insight into the molecular details of YAP/TAZ activity regulation by HERC3, the interplay with the Hippo kinase cascade, the impact of HERC3 on other beta-TRCP substrates and the dependence of the HERC3 carcinogenic role on YAP activity regulation.

Based on the overall interest expressed in the referee reports and the revision outline you provided during the pre-decision discussion, I would like to invite you to submit a revised version of the manuscript in which you address the comments of all reviewers.

From the editorial side, inclusion of a full transcriptome analysis upon HERC3 and YAP/TAZ knockdown as requested by reviewer #2 and addressing their point 4 will not be required for acceptance of the manuscript. Please include the experiments in additional cell lines requested by reviewer #2 in the Appendix of the revised manuscript. Please include the experiments on HERC3 WT and HERC3 CA pro-oncogenic properties in vivo, as requested by reviewer #3 in point 2.

I have extended the resubmission deadline to five months to allow for a more extensive revision as discussed during the pre-decision consultation. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess. Please also see the attached instructions for further guidelines on preparation of the revised manuscript.

Please feel free to contact me if any further questions arise during the revision. Thank you for the opportunity to consider your work for publication. I look forward to receiving the revised manuscript.

Referee #1:

In this manuscript, Yuan and colleagues report a biochemical mechanism in which HERC3, an E3 ligase, promotes YAP/TAZ protein stability. Interestingly, the phenomenon was independent of HERC3 E3 ligase catalytic activity. Interestingly, this regulation seems to be independent of the canonical Hippo-LATS pathway. Finally, they then demonstrate the efficacy of HERC3 in promoting breast cancer tumorigenesis.
The finding is novel, and the results are clear enough to support the authors' claims. However, the overall professionalism of the presented data appears slightly lacking for publication in EMBO Journal in the current form. Thus, to improve the quality of this manuscript, the following are comments the reviewer would like to suggest.

Major Comments:

1. As mentioned in the Discussion, β-TrCP is responsible for degrading many substrates such as YAP, beta-Catenin, Snail, and others. How could the authors be sure that the physiological phenotypes induced by HERC3-mediated β-TrCP inhibition solely depended on YAP/TAZ activity? In Fig. 4I-K, the rescue effect with combined overexpression of TAZ 4SA and YAP 5SA appears too much of an "overkill", since either of the constructs alone is powerful enough oncogenes. Importantly, however, the reviewer is not convinced as to the need to use such powerful mutants of YAP/TAZ. According to this manuscript, HERC3 regulates YAP/TAZ stability via competing with their binding to β-TrCP. Therefore, simply the overexpression of YAP S381A (a phosphodegron leading to recruitment of β-TrCP, leading to its degradation [Zhao et al. Genes & Dev., 2010]) should be sufficient to reverse the effect of HERC3 depletion. On this note, the authors should test whether YAP S381A overexpression reverses the attenuation of tumor formation upon HERC3 depletion in vivo (Fig. 5).
2. The authors did not mention that HERC3 belongs to a family of six related family members in humans (HECR1-6), all of which bear a HECT domain that, according to the findings from this paper, can in theory, all bind to β-TrCP. The reviewer is curious about the functionality of some, if not all, of these isoforms regarding regulating YAP/TAZ stability and/or their relative expression/prognosis in cancers. Also, a previous paper identified the role of Herc4 as an E3 ligase that promotes the degradation of Sav, an upstream component in the Hippo pathway in Drosophila (Aerne et al. PLoS One, 2015). According to the claims made in this paper, Herc4 ultimately promotes YAP/TAZ activation, which is in line with the findings from this manuscript. Perhaps HERC proteins employ diverse mechanisms to strengthen the effect of YAP/TAZ stabilization? These findings should be discussed in the Discussion
3. Information about how the authors came to identify HERC3 as a YAP/TAZ modulator is lacking. Was it a hypothesis-driven approach? Or did the authors perform a screen to identify which E3 ligases modulate YAP/TAZ levels?
4. Although the reviewer acknowledges the existence of an "active YAP" (non-phospho) antibody, it is not yet widely recognized nor used in the field of Hippo-YAP research. The reviewer recommends the additional usage of phospho-YAP antibodies (Ser-127 or Ser-381). In particular, p-YAP (Ser-381) would be facultative for this study since phosphorylation at this site targets YAP for β-TrCP-mediated degradation. Related to this comment, total protein levels should always be checked when using "modification" antibodies; total YAP should also be probed for in Fig. 1A and B.

Minor Comments:

1. The restoration of YAP protein level upon MG132 treatment in HERC3-KD cells is not very clear (Fig. 1F). The blot would benefit from a lighter exposure or quantification across replications.
2. The difference in expression levels of WT and CA HERC3 is too significant in Fig. 2A, whereas in most other cases (e.g. Fig. 2C) the expression appears consistent. Thus, the reviewer recommends repeating Fig. 2A.
3. The authors extensively utilize overexpression constructs. However, the labeling of the proteins on the right side of the blot does not indicate whether the protein of interest is ectopic or endogenous (for example, in FLAG-TAZ expressing cells, the blot is simply labeled "TAZ").
4. In Fig. 3B, the authors claim that HERC3-CA is associated with β-TrCP equally and wild-type, yet the immunoprecipitated β-TrCP amount appears clearly reduced in HERC3-CA. The experiment should be repeated, or the wording should be revised.
5. Statistical analysis should be applied to Fig. 4A
6. The nomenclature used in Fig. 4I-K is ambiguous. shHERC3+4/5 appears as though HERC4 and 5 were knocked down together with HERC3. Instead, the authors should label it as "shHERC3 + TAZ 4SA/YAP 5SA".
7. The difference in Ki67+ intensity presented in Fig. 5F appears relatively marginal. The reviewer recommends displaying a magnified inset and quantifying the data.

Referee #2:

In this manuscript, Yuan et al. report that the HECT domain ubiquitin E3 ligase HERC3 stabilizes YAP/TAZ in TNBC cell lines through direct interaction with b-TRCP, the E3 ligase that mediates YAP/TAZ ubiquitylation. The authors demonstrate that HERC3 binds to the WD40 domain of b-TRCP through its HECT domain, and such interaction prevents b-TRCP1 from binding to YAP/TAZ. The authors also provided evidence that HERC3 silencing in TNBC cells suppressed cell growth and migration both in vitro and in vivo. Analyses of BCa tissue samples and TCGA dataset support a protumorigenic role of HERC3. Most of the data are well controlled and clearly presented to support the major conclusion. The major concern that discourages the publication of this manuscript in its current form is the data are still quite preliminary. For example, transcriptome profiling in

control, shHERC3, shb-TRCP and even shYAP/TAZ cells will be very informative to see how HERC3 regulates the YAP/TAZ pathway. In addition, the limited cell lines used to test the hypothesis and the lack of consideration of other b-TRCP substrates compromised its appeal to a broader audience.

Specific comments:
1. Does HERC3 also prevent b-TRCP from ubiquitylating its other substrates? It will be important for the authors to blot other well-characterized b-TRCP substrates like IkBa and b-catenin in the data where HERC3 expression is modulated. If proven true, does it indicate that HERC3 is a b-TRCP inhibitor?
2. Fig. 1, Does HERC3 differentially regulate non-phosphorylated YAP and p-S127-YAP?
3. Fig. 2-3, is the b-TRCP cDNA used in these experiments b-TRCP1? The authors should test if both b-TRCP1 and b-TRCP2 bind to HERC3.
4. Fig. 3, It will be interesting to test if b-TRCP also suppresses HERC3 E3 ligase activity.
5. Fig. 4, will CA-HERC3 rescue HERC3 knockdown-mediated cell growth/migration inhibition?
6. The authors should test if HERC3-mediated stabilization of YAP/TAZ could also be found in other types of breast cancer cells and even other types of tumor cell lines.
7. Fig. 6A, are "none, mild, moderate, and severe" used to describe tumor stage or HERC3 IHC score? The authors should use the standard BCa stage system to describe the tumor stage and the IHC score to indicate the HERC3 staining intensity.
8. Fig. 6A, is there any correlation between HERC3 expression and BCa stage? The authors are encouraged to analyze both their own staining and the TCGA dataset.
9. Fig. 6, How is the correlation between HERC3 mRNA and protein levels in the CPTAC dataset?
10. Fig. 6, Is YAP/TAZ pathway significantly enriched in HERC3-overexpressed BCa samples?

Minor points:
1. Tumor cell line name should be indicated in the figure panels.
2. The descriptions of the data processing/analyses for Fig. 6 and EV4 are over-simplified, the details, including cohort composition, statistical analysis methods, quantiles, etc. are largely missing.

Referee #3:

The Hippo signaling is responsible for suppressing YAP/TAZ-mediated oncogenic transcriptional program. It was suggested that YAP/TAZ could also be activated without interruption of the Hippo singling, although the mechanism is unclear. In this manuscript, the authors explored a mechanism for Hippo signaling-independent YAP/TAZ activation in breast cancer. They found that an E3 ligase HERC3 can promote YAP/TAZ activation by protecting their degradation. Interestingly, this is independent of the ligase activity of HERC3, but relies on blocking the interaction between beta-TrCP and YAP/TAZ. The study also showed that expression levels of HERC3 correlates with YAP/TAZ protein levels and expression of YAP/TAZ target genes in breast tumor cells and tissues. In addition, knockdown of HERC3 expression inhibits tumorigenesis of breast cancer cells. Overall, the study identified a novel oncogenic role of HERC3 by activating YAP/TAZ. The manuscript was well written with clear presentation of the results. While the conclusion may be interesting to the Hippo field, it should be further examined by addressing the following concerns.

Major concerns:

1. Is the regulation of YAP/TAZ by HERC3 independent of the Hippo pathway? How is it related to the regulation by YAP/TAZ phosphorylation through the Hippo signaling under certain physiological conditions or by cytoskeleton reorganization? In the proposed model (Fig. 6H), when HERC3 is present, YAP/TAZ are protected and enter the nucleus. Does this later step need the Hippo signaling inactivation? It would be important to examine if HERC3 expression and depletion can regulate YAP/TAZ phosphorylation and subcellular localization. The study should also examine if YAP/TAZ phosphorylation or dephosphorylation could antagonize the regulation by HERC3.

2. The study showed that HERC3 is essential to tumorigenic properties of breast cancer cells in vitro and in vivo. Whether the role of HERC3 in these circumstances is through YAP/TAZ is still unclear. While results in Fig. 4I-K could provide some evidence to support this, similar tests by expressing YAP(5SA) or TAZ(4SA) should be performed for the in vivo tumorigenesis and metastasis experiments as well. In addition, the E3 ligase independent feature of YAP/TAZ regulation by HERC3 should be utilized in these in vitro and in vivo tests, because the model predicts that HERC3 (WT) and HERC3(CA) would show similar prooncogenic properties, and this test can help to distinguish the role of HERC3 in regulating YAP/TAZ from other E3 ligase-dependent functions of HERC3, e.g. in regulating SMAD7 or c-Myc.

Minor concerns:

3. In Fig. 1F, while TAZ appears to be fully protected by MG132, it looks like the effect on restoring YAP protein level is quite moderate. How about the effect on active YAP?
4. Although YAP/TAZ degradation was examined in a recombinant system with overexpressing YAP/TAZ, beta-TrCP and

HERC3 (Fig.2E and F), it is important to examine if endogenous YAP/TAZ degradation is prevented or promoted by expressing or silencing HERC3, respectively, using the CHX chase assay.
5. Some data statistics issues need to be resolved. For example, in Fig. 4G, H, and EV3A, replication is needed for statistics. P-value is needed for Fig. 4A and EV3E.
6. In Fig. 4I-K, why both YAP-5SA and TAZ-4SA were expressed together to reverse the HERC3-deficiency? Can either of them do this?
7. In Fig. 6G, in addition to the YAP/TAZ target genes, is there a correlation between HERC3 and YAP/TAZ mRNA expression?
8. Molecular weight markers should be indicated with all of the western blotting results to indicate the relative size of each detected protein.
9. In the experiments for Fig. 1G and 1H, YAP or TAZ was overexpressed. Western blotting results indicating the expression of either protein need to be shown with these two results. Similarly, western blotting results are also needed for Fig. 2B.

Additional suggestions:

10. Some typographical errors, e.g. page 3 " H cells", need to be corrected.
11. In some of the results, e.g. Fig. 1A-C, "active" YAP was indicated along with YAP. How "active" YAP was defined? What antibody was used for this?
12. In Fig. 1G, the bars for Ctrl are not seen, probably due to the scale issue. The reviewer suggests to use two segments for Y axis, so that all bars are visible in the graph.

Referee #1

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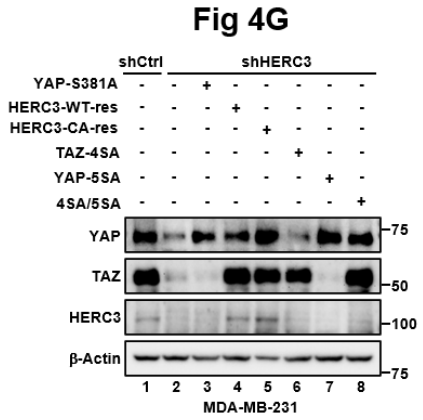
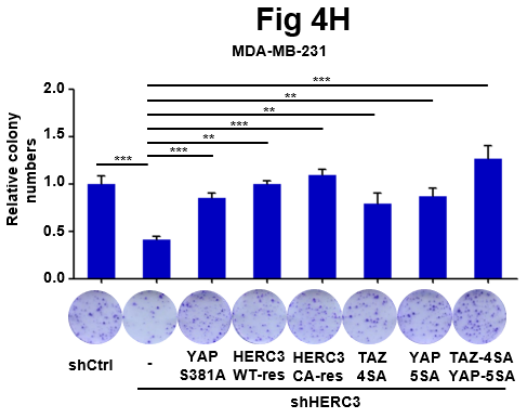
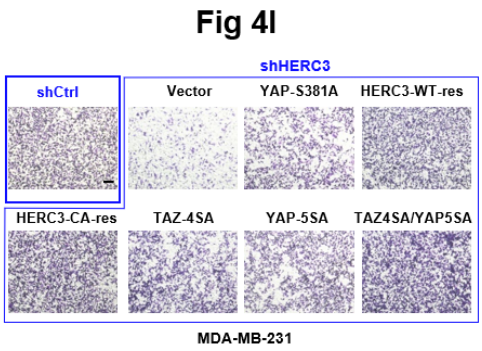
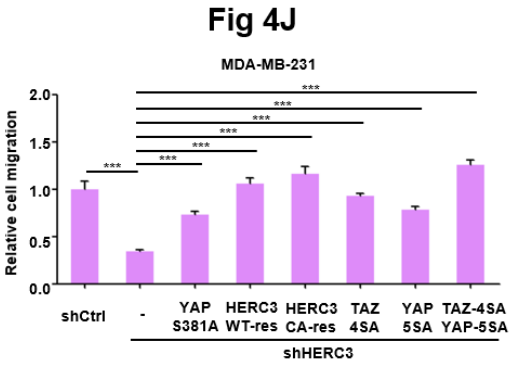
tumor formation upon HERC3 depletion in vivo (Fig. 5).

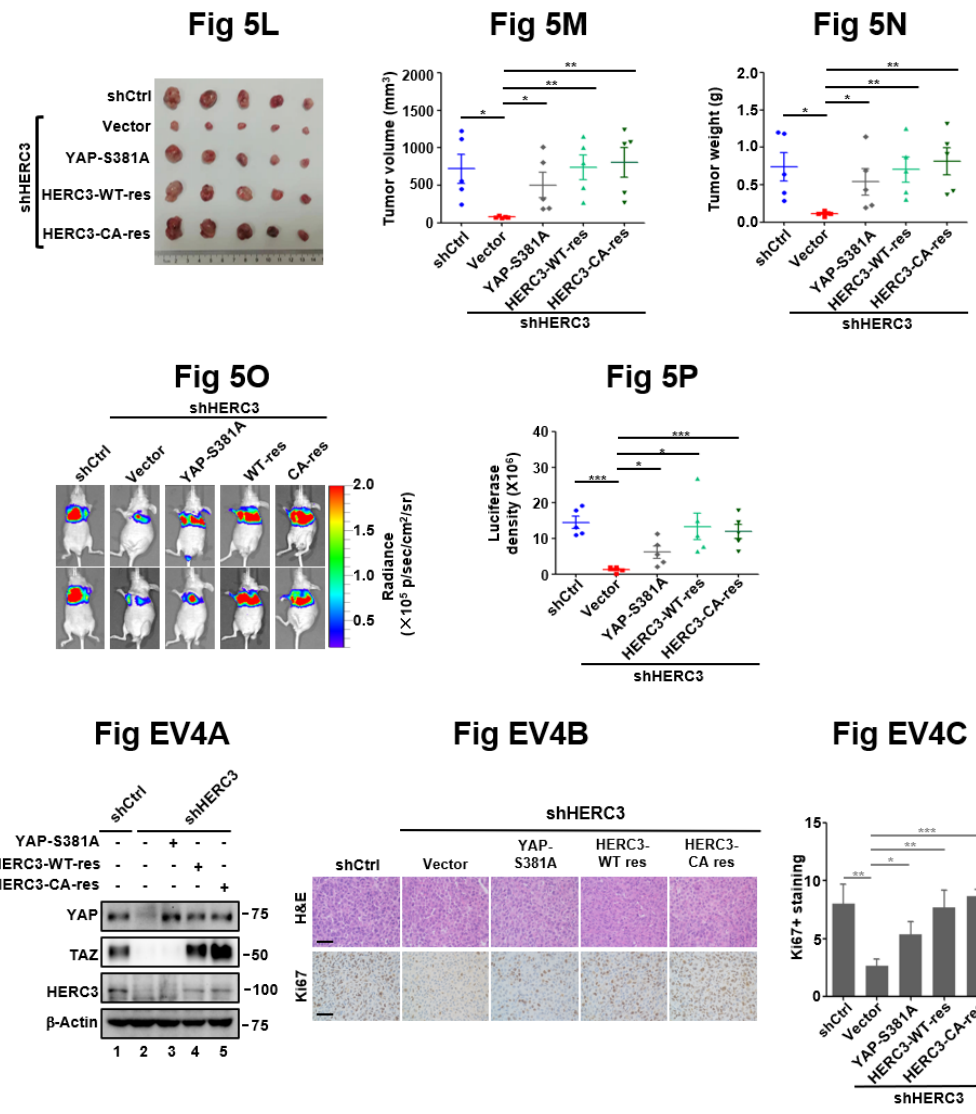
Response: We greatly appreciate and agree the reviewer's constructive suggestion. To determine whether YAP-S381A could counteract the effects of HERC3 depletion, we established ectopic expression of YAP-S381A in HERC3-deficient MDA-MB-231 cells (**Fig 4G**). YAP-S381A partially reversed the inhibitory effect of HERC3 depletion on colony formation (**Fig 4H**) and migration (**Fig 4I** and **J**). Consistently, YAP-S381A also rescued HERC3-deficiency-mediated inhibition of the tumor formation *in vivo* to a certain extent (**Figs 5L-P** and **EV4A-C**).











2. The authors did not mention that HERC3 belongs to a family of six related family members in humans (HECR1-6), all of which bear a HECT domain that, according to the findings from this paper, can in theory, all bind to β-TrCP. The reviewer is curious about the functionality of some, if not all, of these isoforms regarding regulating YAP/TAZ stability and/or their relative expression/prognosis in cancers. Also, a previous paper identified the role of Herc4 as an E3 ligase that promotes the degradation of Sav, an upstream component in the Hippo pathway in Drosophila (Aerne et al. PLoS One, 2015). According to the claims made in this paper, Herc4 ultimately promotes YAP/TAZ

activation, which is in line with the findings from this manuscript. Perhaps HERC proteins employ diverse mechanisms to strengthen the effect of YAP/TAZ stabilization? These findings should be discussed in the Discussion

Response: Survival analysis indicates that elevated expression of HERC3 and HERC4 were negatively correlated with prognosis in TCGA database of breast cancer patients (below). Unlike HERC3, HERC4 did not interact with β-TrCP. Accordingly, depletion of HERC4 had little or no effects on reducing the YAP/TAZ protein levels. Although Aerne and coworkers found that HERC4 promotes SAV degradation and presumably stabilizes downstream Yki, the inability of HERC4 to stabilizes YAP/TAZ in our study suggest two possibilities: 1. The HERC4 effect on Sav is limited to the Drosophila system. We have not gone beyond the scope to test its ability in destabilizing mammalian Sav in mammalian cells. 2. The HERC4 effect on Sav is much less potent than that of HERC3 on directly stabilizing YAP/TAZ. These findings suggest that the HERC3 action on β-TrCP-YAP/TAZ is rather specific.

Figure for reviewers removed

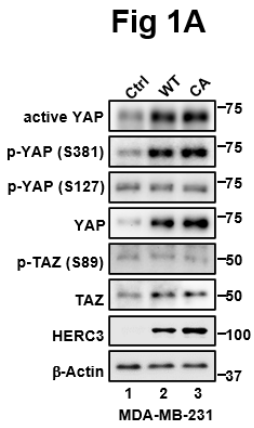
3. Information about how the authors came to identify HERC3 as a YAP/TAZ modulator is lacking. Was it a hypothesis-driven approach? Or did the authors perform a screen to identify which E3 ligases modulate YAP/TAZ levels?

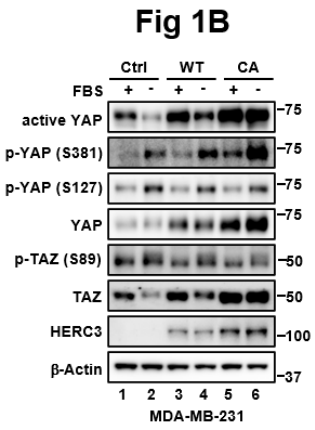
Response: Thanks for pointing this out. We identify HERC3 in a search for ubiquitin E3 ligases that regulate YAP and TAZ activation.

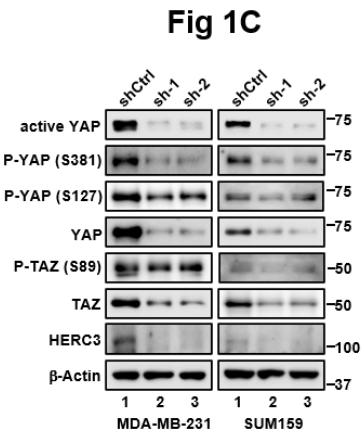
4. Although the reviewer acknowledges the existence of an "active YAP" (non-phospho) antibody, it is not yet widely recognized nor used in the field of Hippo-YAP research. The reviewer recommends the additional usage of phospho-YAP antibodies (Ser-127 or Ser-381). In particular, p-YAP (Ser-381) would be facultative for this study since phosphorylation at this site targets YAP for β-TrCP-mediated degradation. Related to this comment, total protein levels should always be checked when using "modification" antibodies; total YAP should also be probed for in Fig. 1A and B.

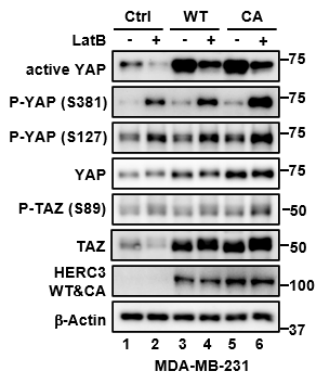
Response: We greatly appreciate the reviewers' comments. Per the reviewer's suggestion, we have revised and included new data on pSer-127 or pSer-381. We

could also observe that HERC3 promoted the stabilization of phospho-YAP/TAZ. Please see **Figs 1A-C** and **EV1A**.







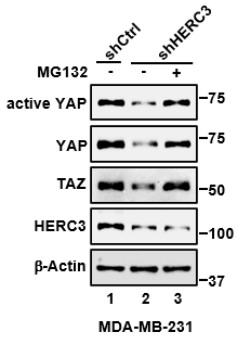


Minor Comments:

1. The restoration of YAP protein level upon MG132 treatment in HERC3-KD cells is not very clear (Fig. 1F). The blot would benefit from a lighter exposure or quantification across replications.

Response: We have redone and replaced with clearer data in **Fig 1F**.

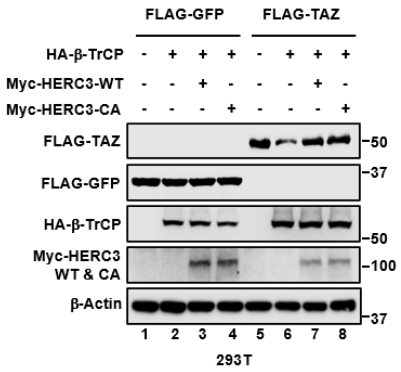
Fig 1F



2. The difference in expression levels of WT and CA HERC3 is too significant in Fig. 2A, whereas in most other cases (e.g. Fig. 2C) the expression appears consistent. Thus, the reviewer recommends repeating Fig. 2A.

Response: Thank the reviewer for his/her kind comments. We have redone and replaced data in **Fig 2A** per the reviewer's suggestion.

Fig 2A



3. The authors extensively utilize overexpression constructs. However, the labeling of the proteins on the right side of the blot does not indicate whether the protein of interest is ectopic or endogenous (for example, in FLAG-TAZ expressing cells, the blot is simply labeled "TAZ").

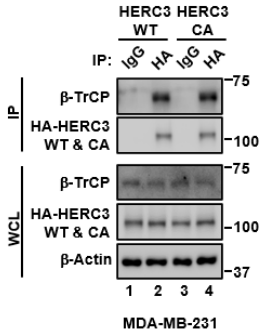
Response: We apologize for the confusion. We have revised the labels of all

figures accordingly.

4. In Fig. 3B, the authors claim that HERC3-CA is associated with β-TrCP equally and wild-type, yet the immunoprecipitated β-TrCP amount appears clearly reduced in HERC3-CA. The experiment should be repeated, or the wording should be revised.

Response: We have redone and replaced the data in **Fig 3B**. The old blot had not been evenly transferred in Western blotting. As shown below, HERC3-WT and HERC3-CA appear to equally associate with β-TrCP.

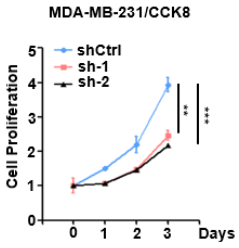
Fig 3B



5. Statistical analysis should be applied to Fig. 4A.

Response: Thanks for pointing this out. It has been revised accordingly (**Fig 4A**).

Fig 4A



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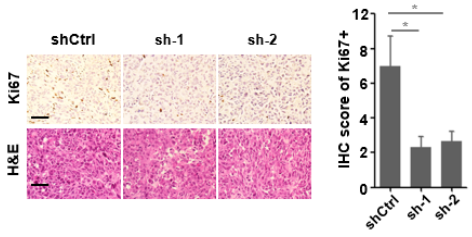
Response: We apologize for the confusion. It has been corrected accordingly.

7. The difference in Ki67+ intensity presented in Fig. 5F appears relatively marginal. The reviewer recommends displaying a magnified inset and quantifying the data.

Response: Thanks for the reviewer's kind suggestion. The data of Fig 5F was in its maximum magnification. We now quantified the data as shown in **Fig 5F** (right).

Fig 5F



Referee #2

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Specific comments:

1. Does HERC3 also prevent b-TRCP from ubiquitylating its other substrates? It will be important for the authors to blot other well-characterized b-TRCP substrates like IkBa and b-catenin in the data where HERC3 expression is modulated. If proven true, does it indicate that HERC3 is a b-TRCP inhibitor?

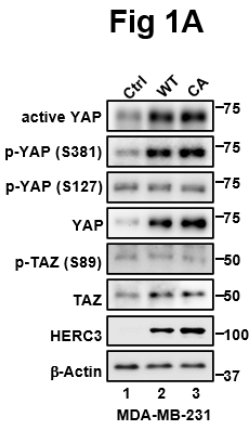
Response: Many thanks for the reviewer's kind comments. We also examined if HERC3 deficiency affects the protein levels of β-catenin, IκBa and Snail, which are substrates of β-TrCP. We found that knockdown of HERC3 led to decreased levels of both β-catenin and Snail proteins, whereas there were no obvious changes in IκBa. We thus believe that HERC3's stabilizing effect is limited to some, not all, of β-TrCP substrates. This is possibly due to distinct yet unclear

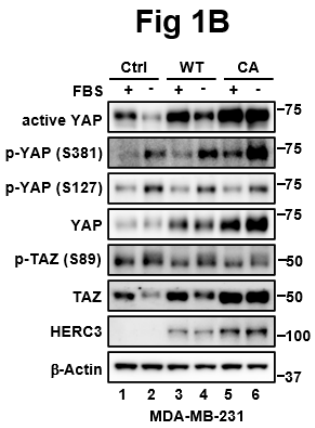
substrate-binding features to β-TrCP. These results are shown below:

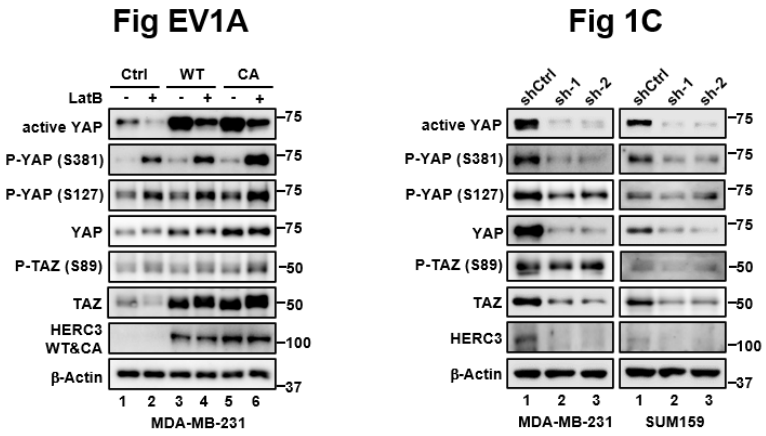
Figure for reviewers removed

2. Fig. 1, Does HERC3 differentially regulate non-phosphorylated YAP and p-S127-YAP?

Response: We believe that HERC3 mainly stabilizes p-S381-YAP, not p-S127-YAP, because it blocks the interaction between β-TrCP and YAP. Indeed, overexpressed HERC3 increased non-phosphorylated YAP (active YAP) expression and has no obvious impact on the level of p-S127-YAP with or without serum starvation or LatB treatment (**Figs 1A-B** and **EV1A**). In addition, depletion of HERC3 remarkably decrease non-phosphorylated YAP (active YAP) level, but did not or weakly affect that of p-YAP (S127) (**Fig 1C**).







3. Fig. 2-3, is the b-TRCP cDNA used in these experiments b-TRCP1? The authors should test if both b-TRCP1 and b-TRCP2 bind to HERC3.

Response: We utilized β-TrCP1 cDNA in the related experiments. To determine whether β-TrCP2 binds to HERC3, we performed co-immunoprecipitation. We found β-TrCP2 also interacted with both HERC3-WT and catalytically inactive mutant HERC3-C1018A.

Figure for reviewers removed

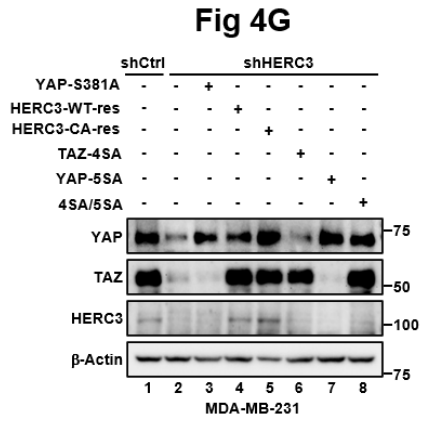
4. Fig. 3, It will be interesting to test if b-TRCP also suppresses HERC3 E3 ligase activity.

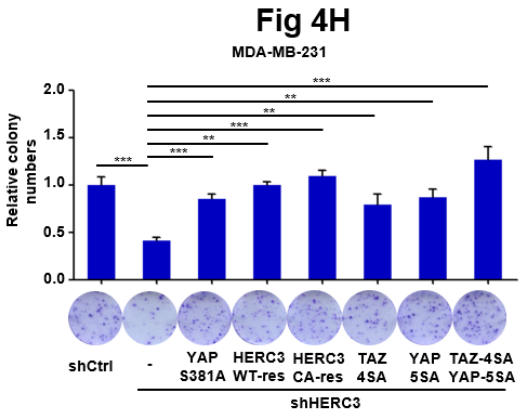
Response: To address the reviewer's question, we determined whether β-TrCP has any effects on HERC3 E3 ligase activity by examining the influence of β-TrCP on HERC3-mediated MM1 degradation. Western blotting analysis showed that HERC3 caused a reduction in the MM1 protein level, which agrees with what a previous study reported (Chen *et al*, 2018. *Cell Death Differ* 25: 2118-2129). Interestingly, β-TrCP suppressed HERC3 E3 ligase activity and rescued the MM1 protein level in a β-TrCP dosage-dependent manner. This is not surprising since β-TrCP can bind to the HECT domain of HERC3 (**Fig 3D** and **E**). β-TrCP might disturb the association between HECT domain and E2 to inhibit HERC3 E3 ligase activity. However, the exact underlying mechanism needs further investigation, which is beyond the topic of our current study.

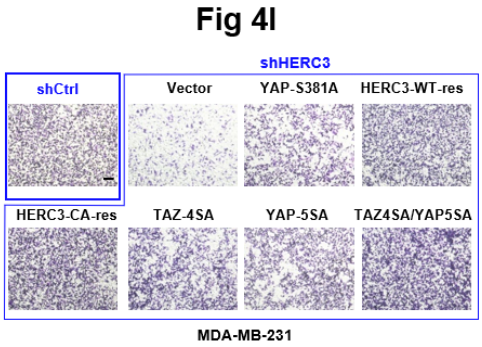
Figure for reviewers removed

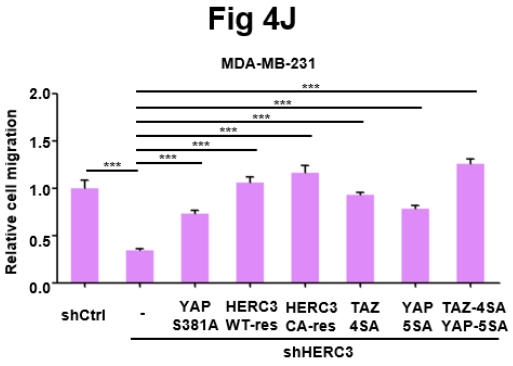
5. Fig. 4, will CA-HERC3 rescue HERC3 knockdown-mediated cell growth/migration inhibition?

Response: To address this issue, we performed colony formation and migration assays. As shown in **Figs 4H-J**, HERC3-CA was able to rescue HERC3-deficiency-induced attenuation of cell colony formation and migration.





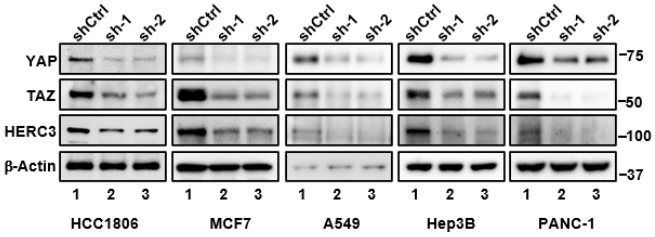




6. The authors should test if HERC3-mediated stabilization of YAP/TAZ could also be found in other types of breast cancer cells and even other types of tumor cell lines.

Response: Per the reviewer's question, we examined a few cancer cell lines. As shown in **Fig EV1D**, depletion of HERC3 also reduced the protein levels of YAP and TAZ in HCC1806 (Triple Negative Breast Cancer), MCF7 (Luminal-A Breast Cancer), A549 (Non-Small Cell Lung Cancer), Hep3B (Liver Cancer) and PANC-1 (Pancreatic Cancer) cells.

Fig EV1D



7. Fig. 6A, are "none, mild, moderate, and severe" used to describe tumor stage or HERC3 IHC score? The authors should use the standard BCa stage system to describe the tumor stage and the IHC score to indicate the HERC3 staining intensity.

Response: We apologize for the confusion. These words were used to describe the IHC score. We have described more accurately in the text.

8. Fig. 6A, is there any correlation between HERC3 expression and BCa stage? The authors are encouraged to analyze both their own staining and the TCGA dataset.

Response: To address this issue, we analyzed both our own staining and TCGA dataset according to the reviewer's suggestion. As shown below, the proportion of elevated HERC3 protein levels in stage II or III of breast cancers (when compared to their paired tissues) was somewhat higher than that of stage I, in tumor samples from hospital (A) and from commercially purchased tissue chips (B). However, there were no changes in the HERC3 mRNA levels from different breast cancer stages of TCGA dataset (C).

Figure for reviewers removed

9. Fig. 6, How is the correlation between HERC3 mRNA and protein levels in the CPTAC dataset?

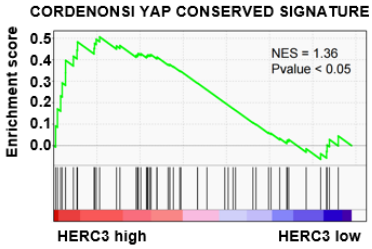
Response: We found no significant correlation between HERC3 mRNA and protein levels of breast cancer in the CPTAC dataset.

Figure for reviewers removed

10. Fig. 6, Is YAP/TAZ pathway significantly enriched in HERC3-overexpressed BCa samples?

Response: To address this issue, we assessed the transcriptome-wide effects of HERC3 on YAP conserved signature genes in TCGA database by GSEA analysis (**Fig EV5D**). The results below revealed that YAP signatures were enriched in breast cancer samples with high HERC3 levels (HERC3 high vs HERC3 low: Normalized Enrichment Score [NES] = 1.36, p value < 0.05).

Fig EV5D



Minor points:

1. Tumor cell line name should be indicated in the figure panels.

Response: Thanks for pointing this out. It has been revised accordingly.

2. The descriptions of the data processing/analyses for Fig. 6 and EV4 are over-simplified, the details, including cohort composition, statistical analysis methods, quantiles, etc. are largely missing.

Response: These have been revised accordingly.

Referee #3

The Hippo signaling is responsible for suppressing YAP/TAZ-mediated oncogenic transcriptional program. It was suggested that YAP/TAZ could also be activated without interruption of the Hippo singling, although the mechanism is unclear. In this manuscript, the authors explored a mechanism for Hippo signaling-independent YAP/TAZ activation in breast cancer. They found that an E3 ligase HERC3 can promote YAP/TAZ activation by protecting their degradation. Interestingly, this is independent of the ligase activity of HERC3, but relies on blocking the interaction between beta-TrCP and YAP/TAZ. The study also showed that expression levels of HERC3 correlates with YAP/TAZ protein levels and expression of YAP/TAZ target genes in breast tumor cells and tissues. In addition, knockdown of HERC3 expression inhibits tumorigenesis of breast cancer cells. Overall, the study identified a novel oncogenic role of HERC3 by activating YAP/TAZ. The manuscript was well written with clear presentation of the results. While the conclusion may be interesting to the Hippo field, it should be further examined by addressing the following concerns.

Major concerns:

1. Is the regulation of YAP/TAZ by HERC3 independent of the Hippo pathway? How is it related to the regulation by YAP/TAZ phosphorylation through the Hippo signaling under certain physiological conditions or by cytoskeleton reorganization? In the proposed model (Fig. 6H), when HERC3 is present, YAP/TAZ are protected and enter the nucleus. Does this later step need the Hippo signaling inactivation? It would be important to examine if HERC3 expression and depletion can regulate YAP/TAZ phosphorylation and subcellular localization. The study should also examine if YAP/TAZ phosphorylation or dephosphorylation could antagonize the regulation by HERC3.

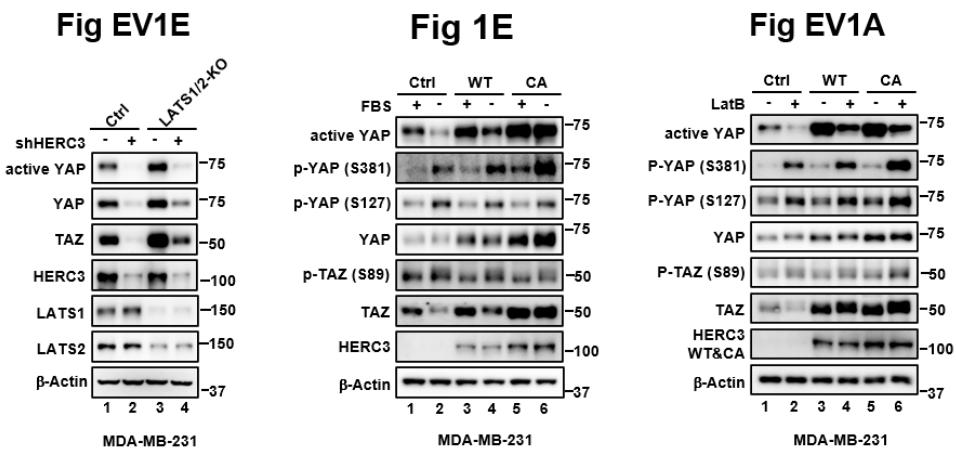
Response: Thanks for the reviewer's suggestive comments. Our study does not intend to address whether HERC3 regulates YAP/TAZ cytoplasmic/nuclear localization, but instead provides an answer as to how HERC3 prevents YAP/TAZ degradation mediated by β-TrCP. Nonetheless, the following data implicate that HERC3 does not regulate YAP/TAZ subcellular localization:

1) HERC3 deficiency could still reduce the YAP/TAZ protein levels in LATS1/2 KO cells (**Fig EV1E**). This suggests that HERC3 blocks other kinases to trigger YAP/TAZ degradation that also involves β-TrCP-mediated ubiquitination.

2) Upon activation of Hippo signaling, HERC3 and HERC3-C1018A profoundly elevated the p-YAP (S381) level, but not p-YAP (S127) and p-TAZ (S89) (**Figs 1E** and **EV1A**). Since phosphorylation of YAP (S127) and TAZ (S89) by LATS result in their 14-3-3-dependent cytoplasmic sequestration, our data suggests HERC3 blocks the degron, not cytoplasmic retention signal, on YAP/TAZ molecules.

3) YAP/TAZ protein levels were decreased in both the cytoplasm and nucleus in HERC3-deficient cells (**Fig 1D**). This again suggests HERC3 blocks the degron of YAP/TAZ, regardless the latter being in cytoplasm or nucleus.

4) Overexpression of HERC3 or HERC3-CA obviously weakened β-TrCP-induced ubiquitination of both YAP and phosphomimetic YAP-S381D.



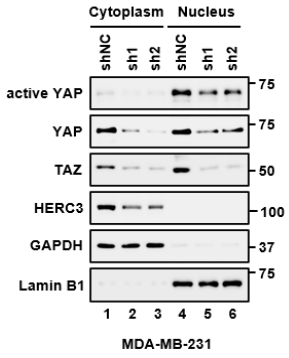
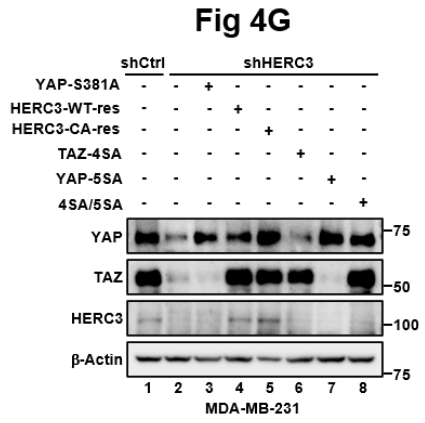
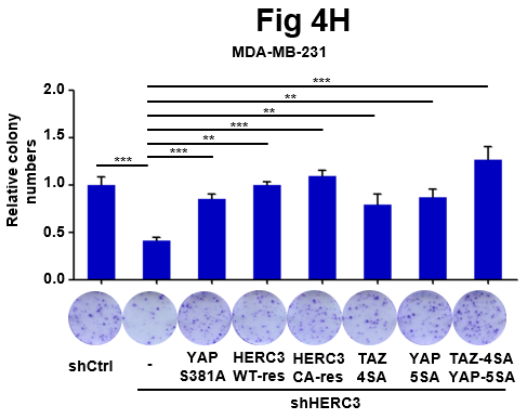


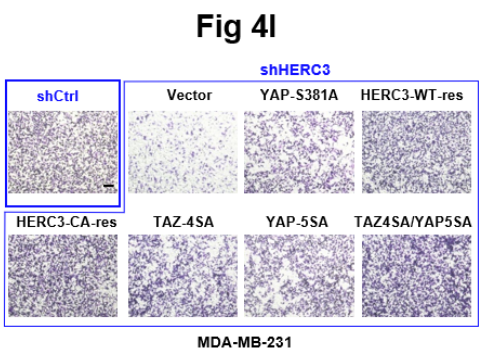
Figure for reviewers removed

2. The study showed that HERC3 is essential to tumorigenic properties of breast cancer cells in vitro and in vivo. Whether the role of HERC3 in these circumstances is through YAP/TAZ is still unclear. While results in Fig. 4I-K could provide some evidence to support this, similar tests by expressing YAP(5SA) or TAZ(4SA) should be performed for the in vivo tumorigenesis and metastasis experiments as well. In addition, the E3 ligase independent feature of YAP/TAZ regulation by HERC3 should be utilized in these in vitro and in vivo tests, because the model predicts that HERC3 (WT) and HERC3(CA) would show similar prooncogenic properties, and this test can help to distinguish the role of HERC3 in regulating YAP/TAZ from other E3 ligase-dependent functions of HERC3, e.g. in regulating SMAD7 or c-Myc.

Response: We can ensure the reviewer that the role of HERC3 in tumorigenesis and metastasis is indeed through YAP/TAZ. To address his/her concerns, we established ectopic expression of YAP/TAZ mutants and HERC3 (WT or catalytically inactive mutant C1018A) in HERC3-deficient MDA-MB-231 cells (**Fig 4G**). TAZ-4SA/YAP-5SA obviously and YAP-S381A partially reversed the inhibitory effect of HERC3 depletion in tumorigenesis and metastasis (**Figs 5L-P** and **EV4**). Additionally, both HERC3-WT and HERC3-C1018A could restore colony formation (**Fig 4H**), cell migration (**Fig 4I-J**) as well as tumor formation and metastasis (**Fig 5L-P** and **EV4**) in the background of HERC3 deficiency. Our observations further confirmed that HERC3 regulated these oncogenic behaviors in a YAP/TAZ dependent manner and independent of its E3 ligase activity.







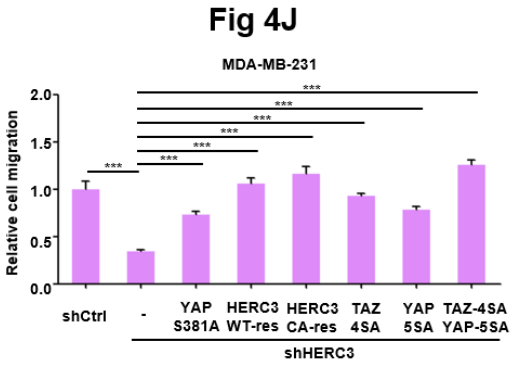


Fig 5L

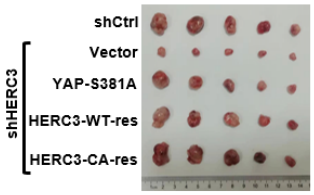


Fig 5M

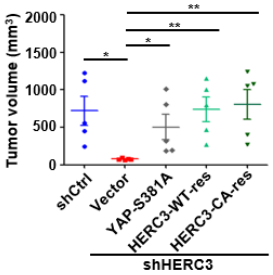


Fig 5N

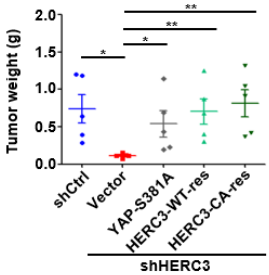


Fig 5O

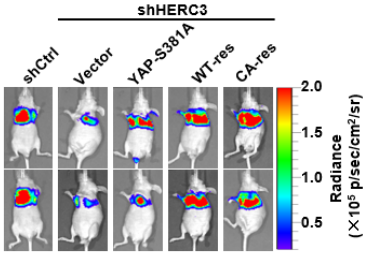


Fig 5P

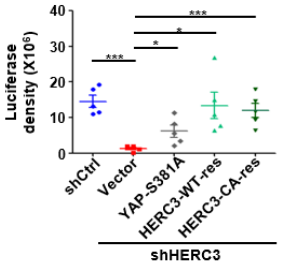


Fig EV4A

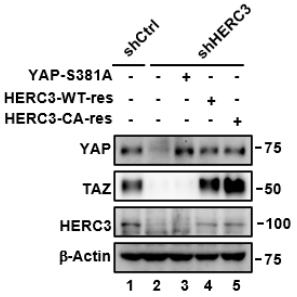


Fig EV4B

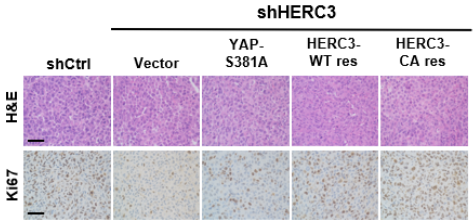


Fig EV4C

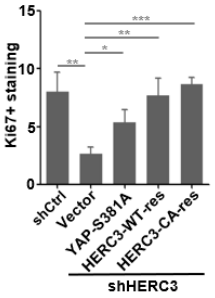


Fig EV4D

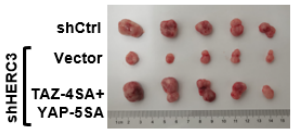


Fig EV4E

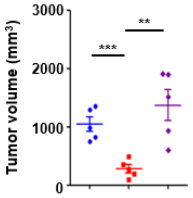


Fig EV4F

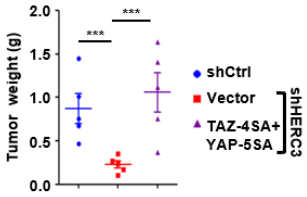


Fig EV4G

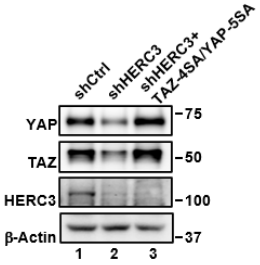


Fig EV4H

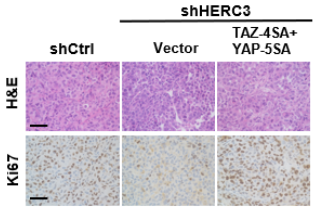
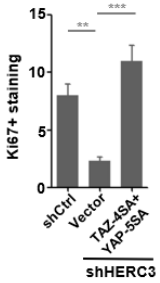


Fig EV4I

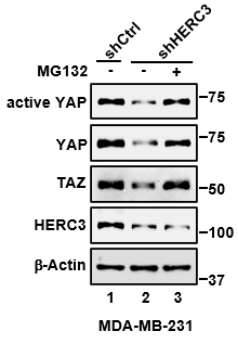


Minor concerns:

3. In Fig. 1F, while TAZ appears to be fully protected by MG132, it looks like the effect on restoring YAP protein level is quite moderate. How about the effect on active YAP?

Response: We have redone this experiment using the active YAP antibody. New data are clearer in **Fig 1F**.

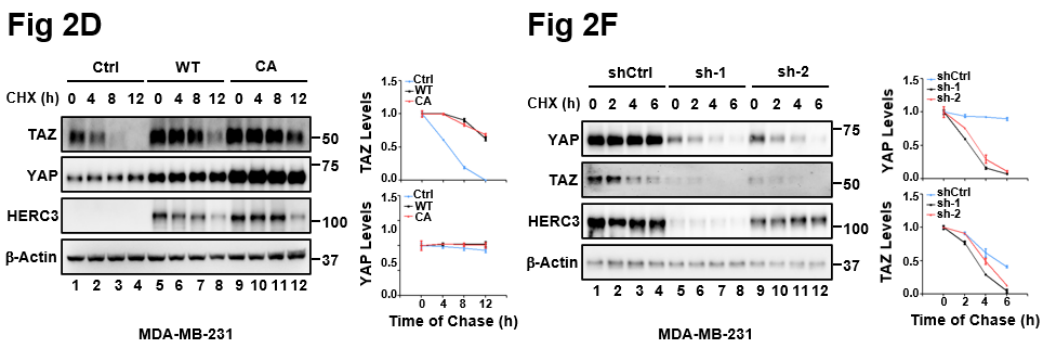
Fig 1F



4. Although YAP/TAZ degradation was examined in a recombinant system with overexpressing YAP/TAZ, beta-TrCP and HERC3 (Fig.2E and F), it is important to examine if endogenous YAP/TAZ degradation is prevented or promoted by expressing or silencing HERC3, respectively, using the CHX chase assay.

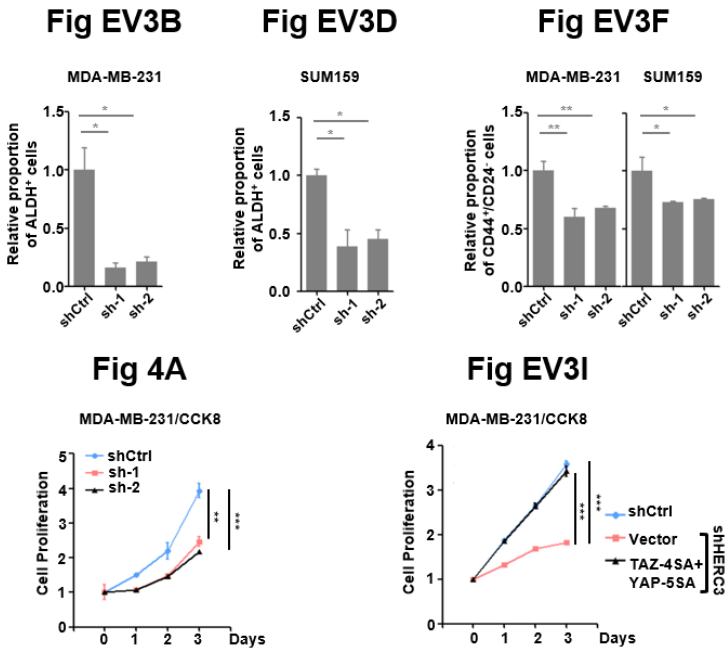
Response: Per the reviewer's instruction, these have been re-done (**Fig 2D** and

F).



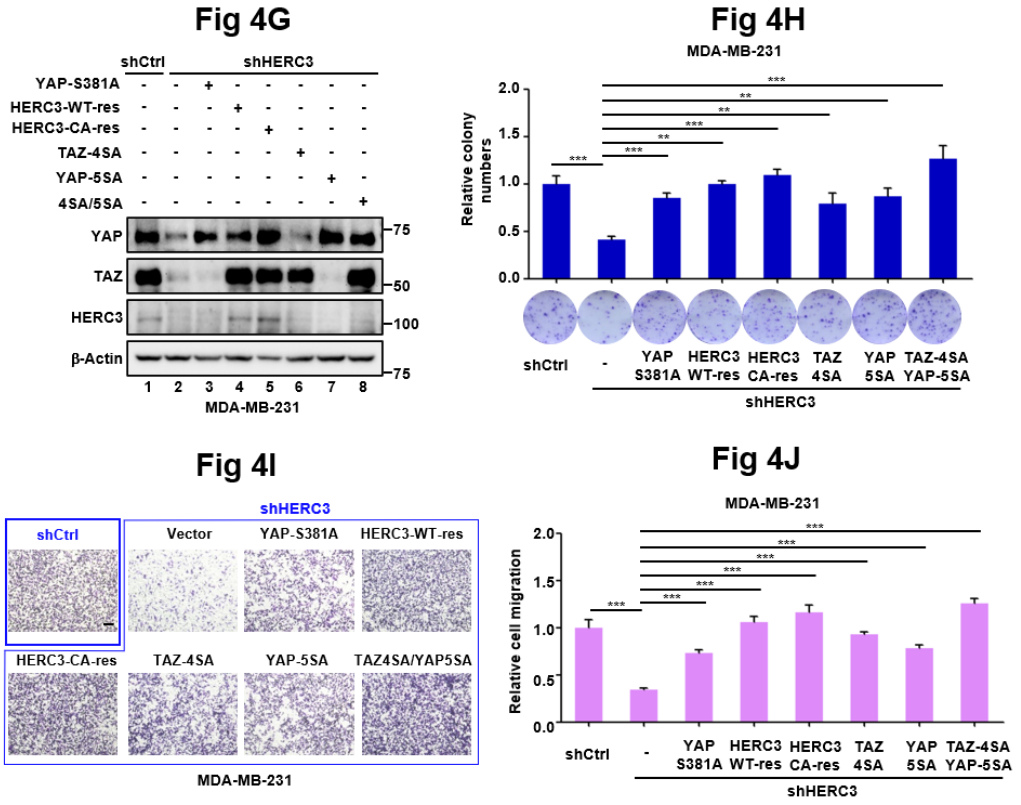
5. Some data statistics issues need to be resolved. For example, in Fig. 4G, H, and EV3A, replication is needed for statistics. P-value is needed for Fig. 4A and EV3E.

Response: Thanks for pointing these out. These have been revised accordingly.



6. In Fig. 4I-K, why both YAP-5SA and TAZ-4SA were expressed together to reverse the HERC3-deficiency? Can either of them do this?

Response: Based on our new data, YAP-5SA or TAZ-4SA could reverse the effects of HERC3 deficiency on cell growth and migration, although the YAP-5SA/TAZ-4SA combo was apparently more potent than the YAP or TAZ alone. Please see **Fig 4G-J**.



7. In Fig. 6G, in addition to the YAP/TAZ target genes, is there a correlation between HERC3 and YAP/TAZ mRNA expression?

Response: To address this point, we carried out Spearman's rank correlation coefficient analysis of TCGA cohort. Results suggest that *HERC3* mRNA level was not correlated with *TAZ* mRNA. Interestingly, there is an apparent positive correlation in the mRNA levels between *HERC3* and *YAP*. This is in line with an early report that *YAP*, not *TAZ*, is one of the transcriptional target genes of *CTGF (iScience, 2020 Jun 26; 23(6):101184).* So there is a YAP-CTGF-YAP positive feedback, where HERC3 stabilizes YAP protein, leading to elevated CTGF

expression that feedbacks to enhance *YAP* mRNA.

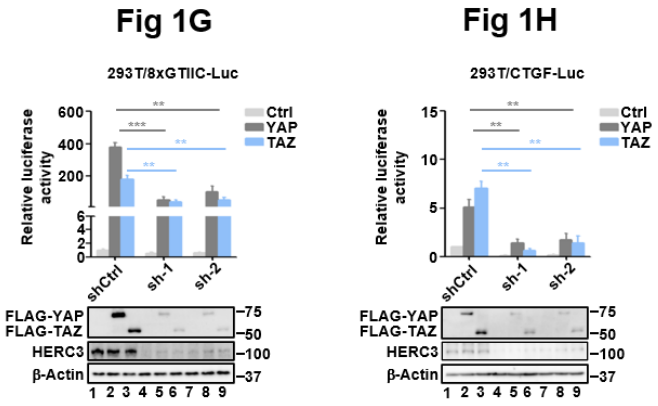
Figure for reviewers removed

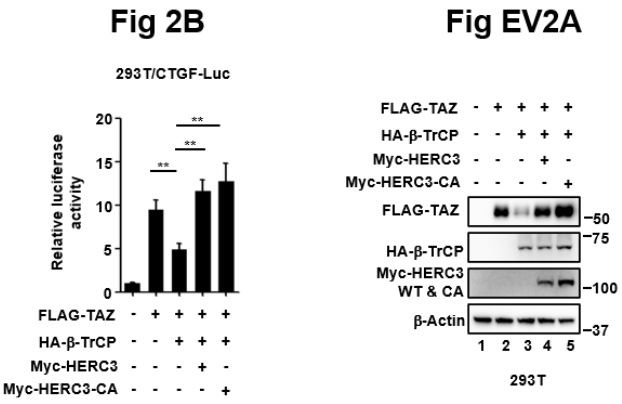
8. Molecular weight markers should be indicated with all of the western blotting results to indicate the relative size of each detected protein.

Response: These have been revised accordingly.

9. In the experiments for Fig. 1G and 1H, YAP or TAZ was overexpressed. Western blotting results indicating the expression of either protein need to be shown with these two results. Similarly, western blotting results are also needed for Fig. 2B.

Response: We now added the Western blot data below the graph. Please see **Figs 1G**, **1H**, **2B** and **EV2A**.





Additional suggestions:

10. Some typographical errors, e.g. page 3 " H cells", need to be corrected.

Response: We apologize for the errors. These have been corrected accordingly.

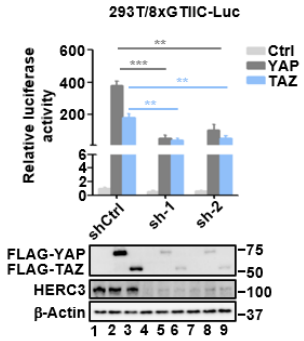
11. In some of the results, e.g. Fig. 1A-C, "active" YAP was indicated along with YAP. How "active" YAP was defined? What antibody was used for this?

Response: The "active" YAP represents the non-phosphorylated form of YAP1. Non-phosphorylated YAP cannot be degraded by the ubiquitin-proteasome system. We used the recombinant anti-active YAP1 antibody [EPR19812] (ab205270) from Abcam.

12. In Fig. 1G, the bars for Ctrl are not seen, probably due to the scale issue. The reviewer suggests to use two segments for Y axis, so that all bars are visible in the graph.

Response: We have replaced data in **Fig 1G** per the reviewer's suggestion.

Fig 1G


293T/8xGTIIC-Luc
Relative luciferase activity
600
400
200
6
4
2
0
**
***
**
**
Ctrl
YAP
TAZ
shCtrl
sh-1
sh-2
FLAG-YAP
FLAG-TAZ
HERC3
β-Actin
75
50
100
37
1 2 3 4 5 6 7 8 9

Thank you for submitting a revised version of your manuscript. Your study has now been seen by all original referees, who find that their major concerns have been addressed and now recommend publication of the manuscript. There now remain only a few editorial issues that have to be addressed before I can extend formal acceptance of the manuscript.

Referee #1:

The revised manuscript by Yuan et al. has addressed our concerns.

I recommend that the article is now suitable for publication in EMBO J.

Referee #2:

The authors did a thorough revision to address all the comments. I would recommend it for publication at Embo J.

Referee #3:

The revised manuscript has addressed this reviewer's concerns. The reviewer suggests including the results and discussion that has been used to address this reviewer's Concern No. 7 to show if there is a correlation between HERC3 and YAP/TAZ mRNA expression in the TCGA cohort.

The authors addressed the minor editorial issues.

Thank you for addressing the final editorial issues. I am now pleased to inform you that your manuscript has been accepted for publication.

EMBO Press Author Checklist

Corresponding Author Name: Xin-Hua Feng
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2022-111549

USEFUL LINKS FOR COMPLETING THIS FORM

The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
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EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January 2022)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Appendix tables

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figures
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Figures
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods; Figure Legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure Legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure Legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
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Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
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Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

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The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	