EMBO *reports*

YAP1-LATS2 feedback loop dictates senescent or malignant cell fate to maintain tissue homeostasis

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Review timeline:

Submission date:	2 August 2017
Editorial Decision:	31 August 2017
Resubmission received:	27 August 2018
Editorial Decision:	4 October 2018
Revision received:	27 November 2018
Editorial Decision:	13 December 2018
Revision received:	22 December 2018
Editorial Decision:	8 January 2019
Revision received:	9 January 2019
Accepted:	10 January 2019

Editor: Achim Breiling/Martina Rembold

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 31 August 2017

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

I am sorry to say that the evaluation of your manuscript is not a positive one. As you will see, while all three referees acknowledge that the findings are potentially interesting, they all raise a number of largely overlapping concerns. The referees indicate that further proof of the senescent phenotype of YAP overexpressing cells is required. Moreover, the referees are concerned about the delayed induction of senescence after 7 passages and question the role of RB in the process and the exact nature of the LATS2-mediated feedback loop. Overall, it appears that none of the referees is fully convinced by the proposed mechanism. Moreover, referee 3 also raises concerns regarding the novelty of the findings.

Due to the nature of the criticisms, the amount of work likely to be required to address them, and the fact that EMBO reports can only invite revision of papers that receive enthusiastic support from the referees upon initial assessment, I am afraid that we do not feel it would be productive to call for a revised version of your manuscript at this stage.

Given the potential interest of your findings, we would, however, have no objections to consider a resubmission of the manuscript in the future if you were able to address all main concerns of the reviewers as highlighted above and in their reports. I would like to stress though that such a manuscript would be treated as a new submission and would be evaluated again, also with respect to the literature and the novelty of your findings at the time of resubmission.

I apologize that I cannot be more positive at this point. I hope, however, that the referee comments are going to be helpful in strengthening your indeed very interesting initial observations and I will be happy to discuss any additional data on this topic with you in the future.

REFEREE REPORTS

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Referee #1:

In this manuscript, Wang and colleagues report a phenomenon in which hyper-activation of the Hippo pathway effector YAP1 induces senescence in the ovarian system, and that deletion of LATS2 with YAP activation results in a switch towards malignant transformation. Conteptually, the authors claim such a negative feedback between LATS2 and YAP1 acts as a homeostatic rheostat for dictating senescent or malignant cell fate.

Although this study is novel and interesting in terms of having characterized a previously overlooked context controlled by Hippo signaling, the conceptual and mechanistic details underlying this phenomenon are not fully explained and the quality of many of the data shown are not very convincing to this reviewer.

To improve the quality of this manuscript, the following are comments the reviewer would like to suggest.

1. As a direct target of YAP, it is known that the expression of Lats2 by YAP can be rapidly induced within several hours (Nature Communications. 2015 10.1038/NCOMMS10186). However, the authors suggest that increased Lats2 by YAP overexpression (S127A) results in cellular senescence following at least seven cell passages. Why does it take so long even though Lats2 is readily upregulated? (i.e. is another factor other than Lats2 involved or is there an alteration in the overall genomic landscape from passage 4 to 7?)

2. From the passage of 7th to 13th, does activity of YAP S127A become transcriptionally inactive? but is the expression of LATS2 enough high to induce senescence? In figure EV2, the authors stated that S127A YAP localized to the cellular cytoplasmic at the 7th passage, but the reviewer is not convinced. The reviewer asks the authors to show how both LATS2 protein expression and YAP nuclear-cytoplasmic localization change in YAP S127A expressing cells among different cell culture passages. Also, compare the other established YAP target genes in each passage.

3. According to the results, the increased LATS2 expression by YAP activation induced senescence via unknown mechanisms (Rb-dependent or Rb-independent). LATS2 deletion suppresses the YAP activation-induced senescence and induces transformation. However, since the extents of YAP activity differentially affect cell fate and phenotypes in the liver when the Hippo components (Sav1, Mst1/2, Nf2, Lats1/2) were mutated (Hippo signaling in the liver regulates organ size, cell fate, and carcinogenesis." Gastroenterology 152.3 (2017): 533-545.). Also it is known that YAP has the five LATS phosphorylation sites. Thus, this reviewer suspects that further activates YAP WT and YAP S127A mutant, which YAP oncogenic activity might overcome YAP-induced senescence and result in malignant transformation.

The reviewer ask the authors to test whether the higher /highest Yap activity (S127/381A mutant and 5SA mutant) induce senescence or transformation. Can this senescent phenotype induced by the hyperactive YAP mutants (2SA and 5SA) be switched to transformation by LATS2 deletion ?

4. Based on cell counting and assessing pRB (807/811) protein levels in Fig. 1-2, the author concluded that cell cycle was arrested in late G1 and S phase by YAP hyperactivation. Since hyperphosphorylation of RB is usually interpreted as a marker of entry into the cell cycle progression and the increase in nuclear size is also commonly observed in YAP-activated cells, the reviewer suspects whether YAP-activated cells may undergo repeated round of endoreplication without proper mitosis instead of cellular senescence. Thus, to rule out the possibility for endoreplication, BrdU incorporation assay is needed to be done in the late passages of YAP-activated cells.

5. The author mainly relied on beta-galactosidase assay and cellular enlargement as a readout of cellular senescence. This needs to be confirmed using other senescence markers like senescence-

associated heterochromatin foci (SAHF), and expression of SASP-related cytokines (e.g. IL6, IL8).

6. Gene editing using CRISPR/Cas9 inevitably requires several rounds of cell passaging to select for a single clone population with verified knockout. If using a pooled population, how can you verify LATS2 was completely knocked out (some cells may have lost only one copy of LATS2)? The methods section of this paper should include this information.

7. The beta-galactosidase assays shown throughout the manuscript are highly inconsistent in terms of cell density. This can pose a problem, since highly confluent cells (or differentiated cells) have been reported to display SA-Bgal activity regardless of whatever genetic alterations have been made. Compare for example, Figure 4C CTRL-hOSE-MXIV micrograph and shRB1-hOSE-YAPS127A micrograph. The difference in cell confluency shown here is too profound to be taken seriously.

8. Increase in LATS protein level in Figure 6A is not convincing. The reviewer suggests the use of LATS1-specific and LATS2-specific primary antibodies.
Also the paper did not mention which LATS antibody was used for Western blotting.

In fact, as far as I knew, there were no such papers to prove that LATS2 antibody can specifically recognize LATS2 protein in immunochemical staining. The authors claimed that LATS2 protein was not detected in LATS2 KO cells (Fig 8D). However, there was no control WT samples that normally express LATS2. Fig 8D indicated that LATS2 seemed to be highly expressed in the stromal cells. The authors also showed the higher intensity of LATS2 immuno-signal in Nevus samples (Fig EV3 C). To make sure these observations, the authors should test whether LATS2 antibody for an immunohistochemistry specifically recognize LATS2 protein by using LATS2 KO cells and WT cells.

Minor comments

1. In the text for Figure 1B, the authors note the trivial effect of singular depletion of either YAP and TAZ on cell proliferation as a result of compensation, yet TAZ protein appears rather slightly decreased compared to control in YAP-depleted cells. Therefore, this explanation is both incorrect in this context and misleading.

2. Numerous typos can be seen throughout the manuscript (e.g Figure 5D is labeled LATS2 KD not KO). Please look over the manuscript carefully.

Referee #2:

The study demonstrated that overexpression of YAP and active YAP-S127A can lead to cell senescence in primary cells, including human ovarian surface epithelial (hOSE) cells, granulosa cells and ovarian microvascular endothelial cells. In addition, knock out LATS2 dampened the YAP induced LATS2 expression, which inhibited YAP induced senescence, and initiated transformation and tumorigenesis. The results suggest that LATS2 and YAP, form a negative feedback loop to regulate YAP-induced senescence. Disruption of the YAP-LATS2 feedback can switch the YAP induced phenotype from senescence to malignant transformation in ovarian cells. It is hard to judge from the experiments whether the LATS2 has a general role in senescence (eg that invoked by other oncogenes like RAS) or if it is restricted to YAP-induced senescence.

Comments
To perform the final experiment properly the authors must compare melanocytes in normal skin to melanocytes in nevi to melanocytes in melanomas. To do this the sections must be stained with melanocyte markers in all samples. Without this comparisons cannot be made, especially to normal skin, as melanocytes are rare in normal skin.
1. In Figure 8D, it needs an independent mark (such as GFP if YAP is GFP-tagged) to show the injected cells of hOSE-LATS2KOYAPS127A in the tumor. YAP staining itself is not a good marker representing injected cells in case there are other types of cells that also show high YAP expression.

2. In Figure EV3, a melanocyte marker should be co-stained with LATS2 in melanoma and Nevus.

Otherwise, conclusion cannot be made with LATS2 staining on its own.

3. It is better for the authors to do a follow up study: keep culturing the hOSE cells transfected with YAP or YAP-S127A after passage 7 when the cells show senescence. Will they die after sustained senescence, or finally recover from senescence? Is YAP induced cell senescence just a transient phenotype?

4. From the literature, LATS2 is actually a direct YAP target gene. Therefore LATS2 is upregulated straight away after YAP overexpression. Why the senescence phenotype of the hOSE cells came up after 5 passages of YAP transfection?

5. Figure 8C LATS2KO&YYAPS127A is supposed to be LATS2KO&YAPS127A; shRB1&YAP127 is supposed to be shRB1& YAPS127A

Referee #3:

Synopsis:
This manuscript describes the role of YAP-LATS2 feedback loop in YAP-induced cellular senescence and malignant transformation. The authors showed that YAP overexpression induces cellular senescence in multiple primary human cells in the presence of LATS2, but not in the absence of LATS2. The authors demonstrated that YAP hyper-activation induces LATS2 expression, constituting a negative feedback loop in the Hippo pathway to prevent malignant transformation. Accordingly, deletion of LATS2 in those cells prevents YAP-induced cellular senescence. Disruption of YAP-LATS2 feedback loop initiates cell transformation and tumorigenesis of primary human ovarian surface epithelial cells.

Comments:
The phenotypic link between cellular senescence and the Hippo pathway (Tschöp et al., Genes Dev. 25, 814-830, 2011; Xie et al., Cancer Res. 73, 3615-3624, 2013 --> although this paper provides alternative results), as well as YAP-induced feedback mechanisms in the Hippo pathway (Moroishi et al., Genes Dev. 29, 1271-1284, 2015; Chen et al., Genes Dev. 29, 1285-1297, 2015; Dai et al., Cell Res. 25, 1175-1178, 2015; Park et al., Oncotarget 7, 24063-24075, 2016) have been reported in previous studies. The current study has some potential significance, but major conceptual advancement is somewhat limited. In addition, there are a number of technical concerns as listed below.

1. The authors generated LATS2 knockout cells by utilizing the CRISPR/Cas9 system. In the Figures 5B and 6D, the authors provided semi-quantitative RT-PCR results indicating that LATS2 mRNA expression is completely abolished in the LATS2-CRISPR-knockout cells. This is a rather strange, indirect, and non-convincing way to confirm a gene inactivation. Genomic sequence of the LATS2 gene needs to be performed to demonstrate the nature of the genetic inactivation of LATS2. Furthermore, Western blotting should be performed to show that LATS2 protein is no longer induced in high passage cells.
2. In Figure 2, the authors claimed that hyper-activation of YAP induces cellular senescence in primary human cells. In contrast, the authors also stated "These data suggest that hOSE-YAP and hOSE-YAP(S127A) cells were arrested in late G1 and S phases (Figure 2C)". Cell cycle arrest and cellular senescence are not same. The authors need to confirm what they observed in YAP-overexpressing cells is truly cellular senescence. More phenotypic characterization (in addition to SA-b-gal staining and WB analysis, such as SAHF formation,,,) may further clarify if these cells are indeed senescent.
3. In all SA-b-gal staining data, the authors need to provide quantitative information.
4. In Figure 4, the authors demonstrated that silencing of RB1 expression prevented YAP-induced cellular senescence. The authors also showed that deletion of LATS2 prevented YAP-induced senescence (Figures 6E¬-G). However, in contrast, overexpression of LATS2 induced cellular senescence both in the presence or absence of RB1 (Figures 7J-L). Those data are not easily reconciled with the authors' model that YAP induces LATS2 expression to induce cellular senescent program (Figure 8). The authors need to provide some explanation or further experimentation to clarify this discrepancy.
5. Figure 6A shows that LATS protein levels were not significantly increased in the YAP

overexpressing cells. This data contradicts to the statement in the text "As shown in Figure 6A, the LATS protein was elevated in hOSE-YAP and hOSE-YAP S127A cells". Importantly, this data argue against a model that LATS2 induction directly contributes to senescence. What is the authors' explanation?
6. Figure 7A, more description is deeded for the LATS2 expressing cells. Is this a stable clone? The expression levels of the ectopic LATS2 should be shown and compared with the endogenous LATS2 level by Western. Are YAP phosphorylation and cytoplasmic localization increased in the LATS2 expressing cells? Figure 7F-H, ectopic expression of LATS2 had little effect on senescence at early passages, indicating that LATS induction is not sufficient to induce senescence. This data needs to be explained.

Resubmission 27 August 2018

Referee #1:

Reviewer's general comment: In this manuscript, Wang and colleagues report a phenomenon in which

hyper‐activation of the Hippo pathway effector YAP1 induces senescence in the ovarian system, and that

deletion of LATS2 with YAP activation results in a switch towards malignant transformation. Conceptually, the authors claim such a negative feedback between LATS2 and YAP1 acts as a homeostatic rheostat for dictating senescent or malignant cell fate.
Although this study is novel and interesting in terms of having characterized a previously overlooked
context controlled by Hippo signaling, the conceptual and mechanistic details underlying this phenomenon are not fully explained and the quality of many of the data shown are not very convincing
to this reviewer. To improve the quality of this manuscript, the following are comments the reviewer would like to suggest.

Authors' response: We thank Reviewer for recognizing the novelty and significance of our research results and below constructive suggestions for us to improve this manuscript.

Reviewer's comment 1. As a direct target of YAP, it is known that the expression of Lats2 by YAP can be
rapidly induced within several hours (Nature Communications. 2015 10.1038/NCOMMS10186). However,
the authors suggest that increased Lats2 by YAP overexpression (S127A) results in cellular senescence
following at least seven cell passages. Why does it take so long even though Lats2 is readily upregulated? (i.e. is another factor other than Lats2 involved or is there an alteration in the overall genomic landscape from passage 4 to 7?)

Authors' response: We thank the reviewer for this insightful question. We conclude that LATS2 plays a critical role in cellular senescence based on our observations that: 1) LATS2 is significantly increased in the replicative senescent hOSE cells. Knockdown of LATS2 in these cells blocked replicative senescence; 2) hyperactivation of YAP1 induced high level of LATS2 and the senescent phenotype in hOSEs, while knockdown of LATS2 in YAP1-hyperactivated hOSE cells blocked YAP1-induced senescence; 3) ectopic expression of LATS2 elicited cellular senescence in primary hOSE cells; 4) ectopic expression of LATS2 induced senescent phenotype in RB1-knockdown hOSE cells. These data provide convincing evidence that hyperactivation of YAP1, via upregulation of LATS2, induces cellular senescence in primary human ovarian surface epithelial cells.

Importantly, we found that hyperactivation of YAP1 was able to induce cellular senescence in a variety of cells, and the time required for induction of senescent phenotypes is cell type-dependent. Notably, YAP1 induced LATS2 expression and cellular senescence in HUVEC cells within 10

days, which is comparable to the time required for constitutively active RAS (HRASG12V) to induce
senescence in other types of cells [*Genes & Development*, 2000, 14:2015–2027; *J Clin Invest.* 2013, 123:4375–4389]. Knockdown of LATS2 in HUVEC cells blocked YAP1-induced senescence, while ectopic expression of LATS2 in HUVEC cells induced senescence (Appendix Fig. S12), indicating that LATS2 is a major player in YAP1-induced senescence in these cells. As indicated in the manuscript, a longer time is needed for YAP1 to induce the senescent phenotype in the cultured hOSEs (~20 days after transfection) and human ovarian granulosa cells (~ 45 days). Therefore, the time for YAP1-induced LATS2 to establish senescence is cell type-specific.

Nevertheless, we agree with the reviewer that LATS2 is not the only factor that is involved in YAP1-induced senescence. As showed in the manuscript, YAP1 also induced upregulation of RB1. The role of RB1 in establishment and maintenance of cellular senescence has been well documented. Moreover, LATS2 was reported to interact with the ATR–Chk1 pathway in the DNA damage response (DDR) [*Oncogene* 2009, 28, 4469–4479; *J. Cell Sci.* 124, 57–67]. Activation of the DDR pathways is involved in both the induction and maintenance of senescence in many cases [*Nature Reviews Cancer* 2008, 8: 512–522; *Cell Cycle* 2007, 6:15, 1831-1836].

Recently, in an effort to understand the mechanism required for the delayed senescent phenotype in the YAP-high hOSEs, we found that hyperactivation of YAP1 induced a significant reduction of deoxyribonucleotide triphosphate (dNTP) levels in the immortalized human OSE cells (Figure R1). Interestingly, recent studies indicated that replication stress-induced decrease in dNTP levels plays a critical role in the establishment of stable oncogene-induced senescence [*Cell Reports* 2015, 11: 893–901; *Cell Reports* 2013, 3: 1252–1265]. At the same time, it showed that expression of a high level of MYC partially blocked senescence induced by dNTP depletion

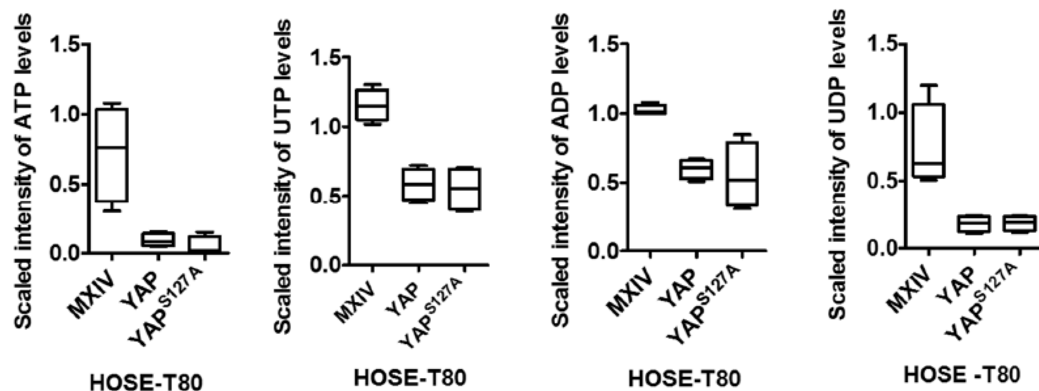

Figure R1. Hyper-activation of YAP1 induces depletion of nucleotides in immortalized OSE cells (HOSET80). Cultured HOSE-MXIV (control), HOSE-YAP and HOSE-YAPS127A cells were harvested when cell reach 75% confluence. Cellular nucleotides were determined using MASS spectrum in Metabolon Inc. The normalized relative levels of ATP, UTP, ADP, and UDP were presented. Each box represents mean ± SEM of 4 samples. Boxes with different letters are significantly different from each other ($P < 0.05$).

[*EMBO J.* 2017, 36: 3409–3420; *Cell Metab.* 2015, 22: 1068 – 1077]. Intriguingly, we found that in early passage hOSE, hyperactivation of YAP1 induced expression of MYC, while in the late passage hOSE cells, hyperactivation of YAP1 reduced expression of MYC (Figure R2, see next page). Therefore, YAP1-induced MYC expression may play a role in delaying LATS2 induced senescence. We believe that more experiments are needed for us to fully understand the molecular mechanisms by which YAP1-LATS2 feedback loop prevents cells from malignant transformation.

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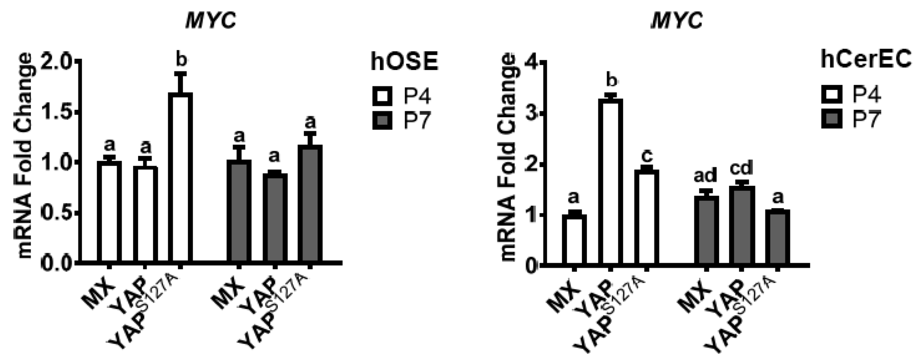

Figure R2. Hyper-activation of YAP1 in hOSE and hCerEC cells induces expression of *MYC* in the low passage cells, but not in the high passage cells. Each bar represents mean + SEM (n=4). Bars with different letters are significantly different from each other ($P < 0.05$, n=4). P4: passage four cells; P7: Passage seven cells.

Reviewer's comment 2. From the passage of 7th to 13th, does activity of YAP S127A become transcriptionally inactive? But is the expression of LATS2 enough high to induce senescence? In figure EV2, the authors stated that S127A YAP localized to the cellular cytoplasmic at the 7th passage, but the reviewer is not convinced. The reviewer asks the authors to show how both LATS2 protein expression and YAP nuclear-cytoplasmic localization change in YAP S127A expressing cells among different cell culture passages. Also, compare the other established YAP target genes in each passage.

Authors' response: We thank the reviewer for the suggestion to improve our manuscript. We analyzed the mRNA and protein expressions of several YAP target genes between Passage 4 and Passage 7. Passage 13 cells with *YAPS127A* cannot be used in these analyses because of severe senescent. As shown in Figure EV2B, *AREG, CCNE*, *MYC*, *HBEGF,* and *ERBB3* were significantly up-regulated in YAP- and YAPS127A expressing hOSE cells at passage 4, suggesting that both YAP1 and YAPS127A are transcriptionally active during this period. Moreover, cells kept growing in passage 4, indicating that these genes may mediate YAP1-induced cell proliferation during early passages. However, when cells were undergoing senescence (passage 7), *AREG*, *CCNE*, *MYC,* and *ERBB3* were no longer up-regulated in YAP- and YAPS127A-expressing cells, indicating that the transcriptional activity of YAP1 and YAPS127A was significantly reduced. Interestingly, LATS2 maintained a high level in both P4 and P7 hOSEs, suggesting that hyperactivation of YAP1 may serve as an initiator of LATS2 expression. After the establishment of senescence, a mechanism other than hyperactivation of YAP1 was active to maintain LATS2, which is essential for the maintenance of cellular senescence.

Although previous data have demonstrated that LATS2 may play a role in cellular senescence, the threshold of LATS2 levels for inducing a senescent phenotype has not been previously documented. We found that during natural replicative senescence, *LATS2* mRNA is about three
hOSE hCerEC
times higher in P13 cells (senescent) than that in P4 cells (non-senescent). Moreover, our *LATS2*-expressing vector increased *LATS2* mRNA levels about four times (compared to basal level), which is sufficient to induce senescence in RB1-knockdown hOSE cells. Since ectopic expression of YAP1 and YAPS127A in hOSEs could elevate the mRNA level of LATS2 about 2.5 to 3.0 times, we believed that it might be enough to induce cellular senescence in hOSEs. Although our evidence showed that YAP and YAPS127A were transcriptionally de-activated in the P7 cells, we found that LATS2 levels were not changed, suggesting that an unknown mechanism exists in the pre-senescent and senescent cells to drive *LATS2* gene expression. The transcriptional regulation of LATS2 in pre-senescent and senescent cells is an open question for us to resolve in the future studies.

Interestingly, we found that in LATS2 intact hOSE-YAPS127A cells, a significant amount of YAP1 protein was localized to the cytoplasm (Figure 1G, Figure EV4 & EV6). However, in the LATS2 knockout hOSE-YAPS127A cells, YAP1 predominantly localized to the nucleus (Figure EV6).

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Obviously, high level of LATS2 in hOSE-YAP and hOSE-YAPS127A cells are actively functioning to suppress YAP1 activity, perhaps via other LATS1/2 phosphorylation sites (As mentioned below, YAP1 protein has 5 LATS phosphorylation sites).

Reviewer's comment 3. According to the results, the increased LATS2 expression by YAP activation induced senescence via unknown mechanisms (Rb-dependent or Rb-independent). LATS2 deletion suppresses the YAP activation-induced senescence and induces transformation. However, since the extents of YAP activity differentially affect cell fate and phenotypes in the liver when the Hippo components (Sav1, Mst1/2, Nf2, Lats1/2) were mutated (Hippo signaling in the liver regulates organ size,
cell fate, and carcinogenesis." Gastroenterology 152.3 (2017): 533-545.). Also it is known that YAP has
the five LATS1/2 phosphorylation sites. Thus, this reviewer suspects that further activates YAP WT and
YAP S127A mutant, which YAP oncogenic activity might overcome YAP-induced senescence and result in
malignant transformation.
The reviewer ask the authors to test whether the higher /highest Yap activity (S127/381A mutant and 5SA mutant) induce senescence or transformation. Can this senescent phenotype induced by the hyperactive YAP mutants (2SA and 5SA) be switched to transformation by LATS2 deletion?

***Authors' response*:** We thank the reviewer for the constructive suggestion.
In senescent hOSE cells, LATS2 and phosphorylated YAP1 (inactive form) was significantly upregulated,
but total YAP level was down-regulated. This observation suggests that YAP1 is inactivated in senescent cells (perhaps via elevated LATS2 in these cells), and that high level of LATS2, not YAP1, is the major regulator of cellular senescence. Consistent with this, both our data in this manuscript and previous reports demonstrate that LATS2 is the major player of cellular senescence. Hyper-activated YAP1 may more likely serve as a trigger (or initiator) of cellular senesce pathway (via upregulating LATS2). From the molecular perspective, LATS2 is a direct target gene of YAP1. Activated YAP1 results in high level of LATS2, which induced the establishment of senescence. According to a recent report (*Genes Dev*. 2015 29: 1271-1284), YAP5SA elevated the expression of LATS2 more than 15 times. Therefore, we believe that YAP5SA
may also induce senescence in hOSE cells and possibly at a faster rate than YAPS127A. Consistent with this hypothesis, we found that ectopic expression of YAP5SA in the hOSE cells induced senescent phenotypes (enlargement of cell size, formation of multiple nuclei, and positive staining of β-Gal) in these cells (Figure R3A & R3B). Moreover, ectopic expression of YAP5SA in LATS2-knockout hOSE cells did not induce significant senescence in these cells, suggesting that LATS2

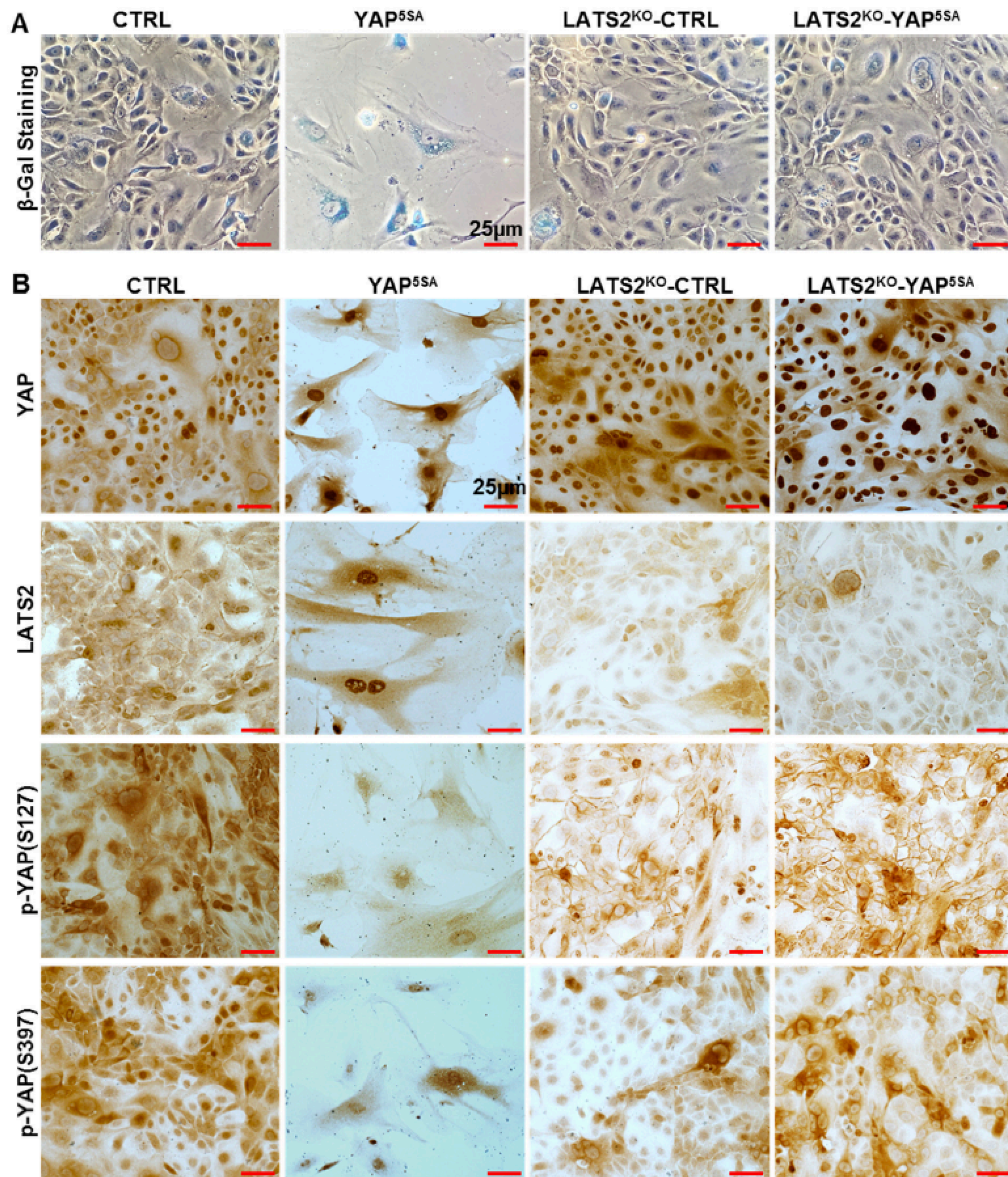

Figure R3. Knockout of LATS2 diminishes senescent phenotype induced by YAP5SA in hOSE cells. **A)** Representative images showing the activity of senescence β-galactosidase (blue color) in hOSE control and hOSE-YAP5SA cells with or without LATS2 deletion (LATS2KO). The primary hOSE cells were transfected with control retroviral vectors or viral vectors expressing YAP5SA at their 2nd passage. Positive cells were selected under 150 μg/ml hygromycin for 6 days. IHC and β-gal staining were performed 12-15 days (2 more subcultures) selection. **B)** Representative images showing expression of YAP1, LATS2, and phosphorylated YAP1 (Ser127 and ser397) in hOSE control (CTRL, transfected with empty vectors) and hOSE-YAP5SA cells (YAP5SA, hOSE cells transfected with vectors expressing YAP1 proteins with all five serine phosphorylation sites mutated) with or without LATS2 gene deletion (LATS2KO). The mutated phosphorylation sites in YAP1 protein of the hOSE-YAP5SA cells are Ser61, Ser109, Ser127, Ser164, and Ser381. Protein levels were determined by immunohistochemistry. Scale bar: 25μm.

deficiency is enough to rescue YAP5SA induced senescence, which further confirmed our conclusion that YAP1-LATS2 feedback loop is critical for maintaining normal cellular function and tissue hemostasis.

Although knockout of LATS2 in hOSE cells also blocked YAP5SA-induced senescence, hOSEYAP5SA-LATS2KO cells grew differently from hOSE-YAPS127A-LATS2KO cells. hOSE-YAP5SALATS2KOcells grew in multiple layers, indicating that these cells are transformed. However, these
cells grew in a growing-dying manner. A portion of cells formed apoptotic bodies and eventually

died in the traditional 2D culture. This cell death may be a consequence of the strong oncogenic stress from the ectopically expressed YAP5SA. Importantly, we realize that YAP1-induced senescence is a cell type-dependent biological event. The detailed mechanism of YAP5SA-induced cell death needs further investigation with different types of cells.

Since genetic and genomic data from TCGA indicates that mutations of YAP1 in the known phosphorylating sites in cancer patients are extremely rare, we are inclined to use wild-type YAP1 or relatively weak mutant YAP1 in our studies so that the experimental results can more appropriately reflect the effect of YAP1 under physiological/pathological conditions.

Reviewer's comment 4. Based on cell counting and assessing pRB (807/811) protein levels in Fig. 1-2, the
author concluded that cell cycle was arrested in late G1 and S phase by YAP hyperactivation. Since hyperphosphorylation of RB is usually interpreted as a marker of entry into the cell cycle progression and
the increase in nuclear size is also commonly observed in YAP-activated cells, the reviewer suspects
whether YAP-activated cells may undergo repeated round of endoreplication without proper mitosis instead of cellular senescence. Thus, to rule out the possibility for endoreplication, BrdU incorporation
assay is needed to be done in the late passages of YAP-activated cells.

Authors' response: We thank the reviewer for this insightful comments.
We agree with the reviewer that hyperphosphorylation of RB1 is usually interpreted as a marker of entry into the cell cycle progression. However, in YAP1-activated hOSEs, the increase of the phosphorylated RB is companied with significant up-regulation of total RB1. This suggested that the level of non-phosphorylated (and/or monophosphorylated) RB1 also significantly increased. Presence of unphosphorylated RB1 drives cell cycle exit and maintains senescence, which is a critical stem for the establishment of stable senescence. In addition, RB1 is not the only senescence-driver induced by *YAP1*. We found that LATS2 expression was also significantly upregulated
by hyperactivation of YAP1 in hOSEs. Most importantly, the senescence of these cells has been confirmed in different dimensions (cell morphology; activation of SA-β-galactosidase, secretion of SASP, formation of SAHF, *etc*.).

To further eliminate the reviewer's concern, we performed BrdU incorporation assay in YAP1-activated cells at their 7th passages. As shown in Appendix Figure S1, hyper-activation of YAP1 inhibits BrdU incorporation in the 7th passage hOSEs at both 6h and 24h groups. Therefore, the endo-replication, if any, should be a very limited event in the high YAP1-induced senescent hOSEs.

We also performed the BrdU incorporation assay in LATS2-KO and shRB groups. As shown in Appendix Figure S7, deletion of LATS2 could rescue YAP-induced inhibition of DNA synthesis in hOSE-YAP and hOSE-YAPS127A cells. More importantly, we found that deficiency of LATS2 cooperated with YAP1 to increase DNA synthesis rate in primary hOSE cells.

Reviewer's comment 5. The author mainly relied on beta-galactosidase assay and cellular enlargement as a readout of cellular senescence. This needs to be confirmed using other senescence markers like senescence-associated heterochromatin foci (SAHF), and expression of SASP-related cytokines (e.g. IL6, IL8).

Authors' response: Following the reviewer's suggestion, we performed IHC analyses for two makers of SAHF (macroH2A and H3K9Me3) (Figure EV1). We also examined expressions of several SASP factors (bFGF, VEGFα, IL6, and IL8) in YAP1-activated hOSE cells at their early (passage 4) and late (passage 7) passages (Figure EV 2). These results further confirmed our finding that hyperactivation of YAP1 induced cellular senescence in primary hOSE cells.

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Reviewer's comment 6. Gene editing using CRISPR/Cas9 inevitably requires several rounds of cell passaging
to select for a single clone population with verified knockout. If using a pooled population, how can you
verify LATS2 was completely knocked out (some cells may have lost only one copy of LATS2)? The methods
section of this paper should include this information.

Authors Response: We thank the reviewer for the constructive suggestion. Yes, we used a pooled population. As shown in our manuscript, almost all cells used in this study were primary cells. Under normal growth conditions, these cells gradually reach senescence (apparent senescent phenotype appears at around the 13th passage for hOSE). Knockdown of LATS2 delays cellular senescence, making it easy to enrich a LATS2-KD cell population. However, it is difficult to have healthy cells as the control (with the same cell density, number of passages and culturing time) to perform a biochemical analysis. To overcome this problem, we purchased a LATS2 sgRNA CRISPR (Vector: pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro) All-in-One Lentivirus set (Catalog Number: K1198315, Applied Biological Materials, Inc.) to knock out LATS2 in these cells. The target sequences and the detailed knockout protocol have been added to the "Materials and Methods" section in the revised version of the manuscript.

Since cells with intact LATS2 grew slowly and gradually became senescent, LATS2 knockout cells rapidly enriched and form a relatively pure LATS2-deficient cell population after puromycin selection. qRT-PCR and semi-quantitative PCR were unable to detect the transcription of LATS2 in these cells, indicating that LATS2 was knocked out in hOSE cells. To further confirm our result, we stained LATS2 and CAS9 in LATS2KO-YAPS127A cell-derived tumor. As shown in Figure 8D, LATS2 immunosignal was detected only in the connective tissues, but not in the tumor cells. We also stained Cas9 in these cells and found that cas9 was highly expressed in nearly all tumor cells, but not in tumor-associated connective tissues (Appendix figure S13). These results demonstrated that LATS2 was knocked out in LATS2KO-YAPS127A hOSE cells.

Reviewer's comment 7. The beta-galactosidase assays shown throughout the manuscript are highly inconsistent in terms of cell density. This can pose a problem, since highly confluent cells (or differentiated cells) have been reported to display SA-bGal activity regardless of whatever genetic alterations have been made. Compare for example, Figure 4C CTRL-hOSE-MXIV micrograph and shRB1-hOSE-YAPS127A micrograph. The difference in cell confluency shown here is too profound to be taken seriously.

Authors' response: We thank the reviewer for pointing this out. Since the growth of hOSEshRB1-YAPS127A cell is much faster than other cells, hOSE-shRB1-YAPS127A cell had higher density after incubating for 72h. We repeated our experiments and replaced the images in the
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figure 4C. We also did SA-β-Gal staining in shRB1-hOSE-MX and shRB1-hOSE-YAPS127A cells and found beta-galactosidase activity was very low in shRB1-hOSE-MX and shRB1-hOSEYAPS127A cells regardless of cell density.

Reviewer's comment 8. Increase in LATS protein level in Figure 6A is not convincing. The reviewer suggests the use of LATS1-specific and LATS2-specific primary antibodies. Also the paper did not mention
which LATS antibody was used for Western blotting.
In fact, as far as I knew, there were no such papers to prove that LATS2 antibody can specifically recognize LATS2 protein in immunochemical staining. The authors claimed that LATS2 protein was not
detected in LATS2 KO cells (Fig 8D). However, there was no control WT samples that normally express
LATS2. Fig 8D indicated that LATS2 seemed to be highly expressed in the stromal cells. The authors also

showed the higher intensity of LATS2 immuno-signal in Nevus samples (Fig EV3 C). To make sure these
observations, the authors should test whether LATS2 antibody for an immunohistochemistry specifically
recognize LATS2 protein by using LATS2 KO cells and WT cells.

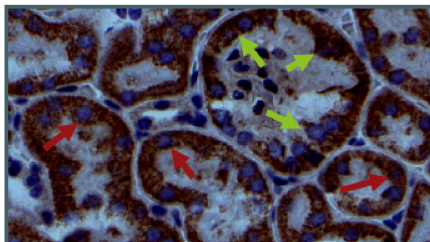
Authors' response: We thank the reviewer for the constructive suggestions. As mentioned by the reviewer, there are no commercial antibodies that specifically detect LATS2 using Western blotting. The LATS antibody used in Western blotting analyses in the present study was from Cell Signaling Technologies (CST#3477), a monoclonal antibody (C66B5) that is able to detect both LATS1 and LATS2.

The additional three antibodies available in our laboratory, which were purchased from the Cell Signaling Technologies (CST#5888), Sigma (PLA0110), and Abcam (ab110780), could not recognize LATS2 protein in the Western blotting analyses, although they were claimed to be LATS2-specific by vendors.

Fortunately, we found that the LATS2 antibody from the Cell Signaling Technologies (CST#5888) could be used to specifically recognize human LATS2 protein in immunohistochemistry. The specificity of this antibody was validated using LATS2 conditional knockout mouse kidney tissues, in which floxed LATS2 was knocked out by a PAX8-driven CRE. As shown in Figure R3, in the control (PAX8-CRE mice) kidney tissue, LATS2 immunosignal (brown color) was detected in PAX8 positive renal tubular epithelial cells and some of PAX8 negative parietal epithelial cells (Figure R4A, see next page). However, in kidney tissues derived from the PAX8-CRE-LATS2flox/flox
mice, LATS2 immunosignal disappeared in epithelial cells that express PAX8, but strong LATS2 immunosignal are still detected in the PAX8 negative epithelial cells (Figure R4B, see next page). This data indicated that antibody CST#5888 can specifically detect LATS2 in FFPE sections.

In addition, this antibody detects LATS2 in P13 hOSE cells, but not in LATS2 knockout cells (hOSE-LATS2KO cells) in an IHC assay (Figure R3, supplementary Fig. S11 & S12). This body of evidence, together with data presented in the manuscript, indicates that the LATS2 antibody used in the IHC study specifically recognizes LATS2.

A) LATS2 in PAX8-CRE



B) LATS2 in PAX8-LATS2 KO

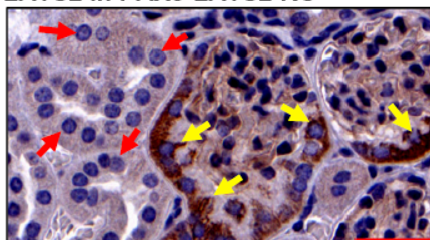


Figure R4. Expression of LATS2 protein in the renal epithelial cells of the wild-type (PAX8-CRE) and LATS2 knockout (PAX8-LATS2 KO, driven by PAX8-CRE) mice.

LATS2 immunosignal (brown color) was detected in the epithelial cells of the wild-type kidney, which have LATS2 expression. However, in PAX8 positive tubal epithelial cells of the LATS2 knockout kidney (Red arrow pointed area), LATS2 immunosignal disappeared, while LATS2 signal was still apparent in the PAX8 negative epithelial cells (yellow arrow pointed area). This data indicated that this antibody (CST#5888) can specifically detect LATS2 in FFPE sections. Scale bar: 100 µm.

Minor comments

Reviewer's minor comment 1. In the text for Figure 1B, the authors note the trivial effect of singular

depletion of either YAP and TAZ on cell proliferation as a result of compensation, yet TAZ protein appears rather slightly decreased compared to control in YAP-depleted cells. Therefore, this explanation is both incorrect in this context and misleading.

Authors' response: We thank the reviewer for the comment. Compensation and counteraction between YAP and TAZ were reported by several research groups [*Nature Communications 2017,* 8**:** 15161; *Development* 2015, 142: 3021–3032; *J Biol Chem*.2015, 290: 27928-27938]. In the human OSE cells, knockdown of either YAP or TAZ alone has a limited effect on cell proliferation. However, YAP and TAZ double knockdown totally blocks cell proliferation, indicating that YAP and TAZ have compensating effects on hOSE cell proliferation. Although TAZ protein was slightly decreased in YAP-knocked down cells, that slight reduction of TAZ protein did not compromise hOSE cell proliferation. Whether the expression of YAP and TAZ in hOSE cells are also regulated in a compensatory manner is not documented. Our preliminary studies showed that the expression of these two transcription activators might not be strictly compensated. The manuscript has been revised accordingly.

Reviewer's minor comment 2. Numerous typos can be seen throughout the manuscript (e.g Figure 5D is labeled LATS2 KD not KO). Please look over the manuscript carefully.

Authors' response: We appreciate your careful review of our manuscript. We thoroughly edited our manuscript. Two senior scientists did proofreading before the resubmission.

Referee #2:

Reviewer's general comment: The study demonstrated that overexpression of YAP and active YAPS127A
can lead to cell senescence in primary cells, including human ovarian surface epithelial (hOSE) cells, granulosa cells and ovarian microvascular endothelial cells. In addition, knock out LATS2 dampened the YAP induced LATS2 expression, which inhibited YAP induced senescence, and initiated
transformation and tumorigenesis. The results suggest that LATS2 and YAP, form a negative feedback
loop to regulate YAP-induced senescence. Disruption of the YAP-LATS2 feedback can switch the YAP
induced phenotype from senescence to malignant transformation in ovarian cells. It is hard to judge from the experiments whether the LATS2 has a general role in senescence (eg that invoked by other oncogenes like RAS) or if it is restricted to YAP-induced senescence.

Authors' response: We thank Reviewer for the comment. Our present data show that LATS2-induced senescence is critical for the YAP1-LATS2 feedback loop to maintain ovarian tissue homeostasis. Moreover, we found that LATS2 was elevated in natural replicative-triggered senescence in hOSE cells. Deletion of LATS2 in ovarian cells using CRISPR/Cas9 system not only prevented YAP1-induced senescent phenotypes, but also delayed natural replicative cellular senescence, suggesting that LATS2 is not only involved in YAP1-induced senescence, but also in the natural replicative senescence. Previous studies also showed that LATS2 was required in RB1-overexpressing-induced senescent phenotype [*Genes Dev.* 2011, 25: 814–830]. In addition, Aylon et al. reported that cells escaped from RAS-induced senescence showed markedly reduced levels of LATS2 and enhanced hypermethylation of the LATS2 gene promoter [*Oncogene* 2009, 28*:* 4469–4479]. These results suggest that the role of LATS2 in cellular senescence is not limited to the ovarian cells and YAP1-induced senescence.

Reviewer's major comment:
To perform the final experiment properly the authors must compare melanocytes in normal skin to melanocytes in nevi to melanocytes in melanomas. To do this the sections must be stained with melanocyte markers in all samples. Without this comparisons cannot be made, especially to normal skin,
as melanocytes are rare in normal skin.

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Authors' response: Since our antibody is only suitable for IHC analysis with FFPE tissue, we have difficulty to co-localize the melanocyte marker with LATS2. However, we can identify melanocytes using the architecture context and cellular morphologic features. Typically, the major feature of the melanocytes includes: 1) oval or fusiform, dendritic cells, smaller than keratinocytes; 2) reside in the basal layer of epidermis; 3) the cytoplasm of melanocytes are intensely white due to a high refractive index of melanin. We stained LATS2 in a commercially available melanoma and Nevus tissue array (SK181, US Biomax Inc) and found that melanocytes had no LATS2 expression. This result is consistent with the LATS2 expression data reported by the Human Protein Atlas (https://www.proteinatlas.org/). Results extracted from the Human Protein Atlas database also clearly demonstrate that keratinocytes express LATS2 protein, but melanocytes have no (or very low, if any) expression of LATS2 protein (Figure R5). The antibody used in their study (HPA039191) has been validated by the Human Protein Atlas.

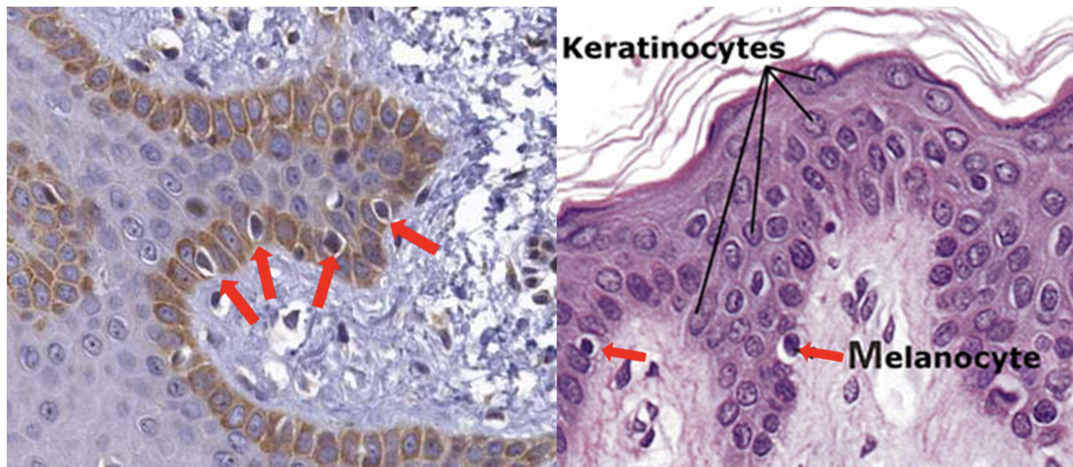

Figure R5. Human melanocytes do not express LATS2. Representative images showing expression of LATS2 in human skin cells detected by immunohistochemistry (IHC). Please note that Keratinocytes are LATS2 (brown color) positive, while melanocytes (red arrows pointed cells) are LATS2 negative. Data were extracted from the Human Protein Atlas database (https://www.proteinatlas.org/). The LATS2 antibody (HPA039191) has been validated by the Human Protein Atlas. Images were used by permission.

Reviewer's comment 1. In Figure 8D, it needs an independent mark (such as GFP if YAP is GFP-tagged) to show the injected cells of hOSE-LATS2KOYAPS127A in the tumor. YAP staining itself is not a good marker representing injected cells in case there are other types of cells that also show high YAP expression.

Authors' response: We thank the reviewer for the constructive suggestion. The vectors used for deletion of LATS2 and ectopic expression of YAPS127A have no GFP tag. To address the reviewer's concern, we stained CAS9 protein in the tissues using immunofluorescence assay. As shown in Figure R6, almost all tumor cells are CAS9 positive, suggesting that this tumor was derived from hOSE-LATS2KO-YAPS127A cells.

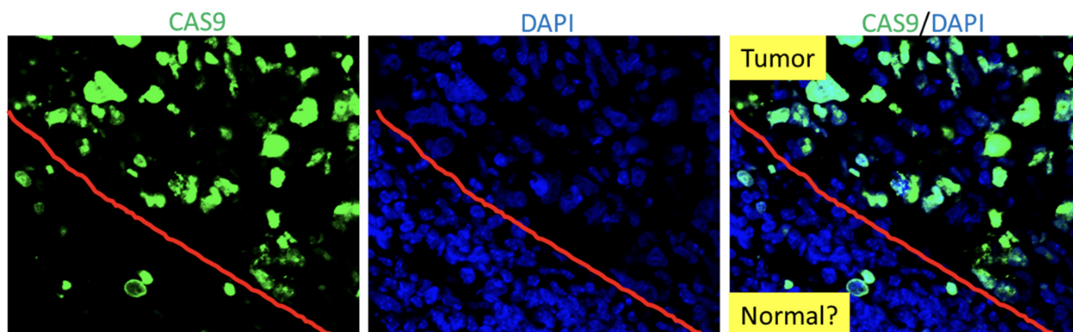

Figure R6. Fluorescent immunohistochemistry detection of CAS9 in the xenograft tumor cells. The CAS9 immunosignal was visualized by an Alexa-488 (green)-conjugated second antibody. Nuclei were

stained with DAPI. These data showed that the tumor cells are derived from hOSE-LATS2KOYAP1S127A cells. Scale bar: 20µm.

Reviewer's comment 2. In Figure EV3, a melanocyte marker should be co-stained with LATS2 in melanoma and Nevus. Otherwise, conclusion cannot be made with LATS2 staining on its own.

Authors' response: As mentioned above, we stained LATS2 in a commercially available melanoma and Nevus tissue array (SK181, US Biomax Inc). Since this antibody for LATS2 protein analysis is only suitable for IHC analysis with FFPE tissue, it's challenging to co-localize the melanocyte marker with LATS2 in FFPE tissue. However, we can identify melanocytes using the architecture context and cellular morphologic features. Typically, mature melanocytes are oval or fusiform and reside in the basal layer of the epidermis. When scrutinized through a conventional microscope, melanocytes appear as "clear cells" (although they are not clear cells) in and immediately beneath the row of epidermal basal cells. The staining results showed that these cells had no or very low (if any) expression of LATS2 (Figure R5).

Reviewer's comment 3. It is better for the authors to do a follow up study: keep culturing the hOSE cells transfected with YAP or YAP-S127A after passage 7 when the cells show senescence. Will they die after sustained senescence, or finally recover from senescence? Is YAP induced cell senescence just a transient phenotype?

Authors' response: We thank the reviewer for the provoking questions. We did the experiment suggested by the reviewer and found that the hOSE-YAP and hOSE-YAPS127A cells survived several months without division in the growth medium. However, sub-culture was very difficult for these cells because, for every sub-culture, a portion of trypsinized cells couldn't reattach to the culture dishes. Successfully attached cells lost their dividing capability, but were viable in the culture dishes for several months. The long-term incubation increased the portion of cells with enlarged cell size, nuclear heteromorphism, and multinucleated. These observations indicated that hyperactivation of YAP1 induced permanent cell cycle arrest in these cells.

Reviewer's comment 4. From the literature, LATS2 is actually a direct YAP target gene. Therefore LATS2 is
upregulated straight away after YAP overexpression. Why the senescence phenotype of the hOSE cells
came up after 5 passages of YAP transfection?

Authors' response: As mentioned above (authors' response to comment #1, reviewer #1), cell type is an important factor that affects the time of senescence initiation and establishment. For example, endothelial cells (HOMEC and HUVEC) are very sensitive to YAP1 hyper-activation, and the senescent phenotype can be observed rapidly (within two passages sub-culturing, about 10 days after YAP1S127A transfection). Ovarian cells (hOSE and hGC) are relatively resistant to YAP1-induced senescence (Most YAP-overexpressed hOSE cells did not have significant senescent phenotype until sub-culturing for ~ 20 days).
Our recent data showed that there is an overall alteration in the genomic landscape during the establishment of YAP1-induced senescence. Nevertheless, knockdown of LATS2 blocked natural replicative and YAP1-induced senescence clearly indicate the key role of LATS2 in this process. Other potential mechanism(s), such as the interaction between YAP1-induced MYC expression and nucleotide depletion, has been discussed in page 2 – page 3 of this response letter.

Reviewer's comment 5. Figure 8C LATS2KO&YYAPS127A is supposed to be LATS2KO&YAPS127A; shRB1&YAP127 is supposed to be shRB1& YAPS127A

Authors' response: We thank the reviewer for his/her careful review of our manuscript. This has been corrected in the revised manuscript.

Referee #3:

Synopsis: This manuscript describes the role of YAP-LATS2 feedback loop in YAP-induced cellular

senescence and malignant transformation. The authors showed that YAP overexpression induces cellular senescence in multiple primary human cells in the presence of LATS2, but not in the absence of LATS2.
The authors demonstrated that YAP hyper-activation induces LATS2 expression, constituting a negative feedback loop in the Hippo pathway to prevent malignant transformation. Accordingly, deletion of LATS2 in those cells prevents YAP-induced cellular senescence. Disruption of YAP-LATS2 feedback loop initiates cell transformation and tumorigenesis of primary human ovarian surface epithelial cells.
The phenotypic link between cellular senescence and the Hippo pathway (Tschöp et al., Genes Dev. 25, 814-830, 2011; Xie et al., Cancer Res. 73, 3615-3624, 2013 --> although this paper provides alternative results), as well as YAP-induced feedback mechanisms in the Hippo pathway (Moroishi et al., Genes Dev. 29, 1271-1284, 2015; Chen et al., Genes Dev. 29, 1285-1297, 2015; Dai et al., Cell Res. 25, 1175-1178, 2015; Park et al., Oncotarget 7, 24063-24075, 2016) have been reported in previous studies. The current study has some potential significance, but major conceptual advancement is somewhat limited. In addition, there are a number of technical concerns as listed below.

Authors' response: We thank the reviewer for pointing out the importance of our study. Several relevant papers published in *Genes & Development* recently further indicated the significance of the present study.

We agree with the reviewer that several recent publications have reported some fragmented information considering the interaction between YAP1 and LATS2 and the role of LATS2 in cellular senescence. For example, Xie *et al.* observed that YAP1 inhibited cell senescence [*Cancer Res.* 2013, 73: 3615-3624]. Their conclusion was inconsistent with our observation that YAP1 induced senescence in cultured primary cells. We believe that they observed the phenotype that we have identified in the early passage cells (less than 5 passages), in which activation of YAP1 stimulates cell growth and promotes the cell cycle. Guan *et al.* observed YAP1 stimulated LATS2 expression in immortalized cell lines, but they did not provide any evidence to show that YAP1-induced LATS2 serves as a sensor of oncogenic stress and the sentinel of cell fate (to senescence or malignant transformation). In the present study, we systematically established the role of YAP1-LATS2 feedback loop in the homeostasis of ovarian tissues (potentially other tissues). Our work provides systematic evidence to show that the YAP1-LATS2 feedback loop acts as a homeostatic rheostat dictating cell fate (senescence VS malignant transformation). Most importantly, the present studies provide direct evidence to show that dysfunction of the YAP1-LATS2 feedback loop is sufficient to induce malignant transformation and tumorigenesis. Importantly, we provide the first evidence to unveil the clinical significance of LATS2-driven senescence in human disease (nevi). From this perspective, this manuscript presents important findings with novelty and clear clinical relevance.

Reviewer's comment 1. The authors generated LATS2 knockout cells by utilizing the CRISPR/Cas9 system.
In the Figures 5B and 6D, the authors provided semi-quantitative RT-PCR results indicating that LATS2 mRNA expression is completely abolished in the LATS2-CRISPR-knockout cells. This is a rather strange, indirect, and non-convincing way to confirm a gene inactivation. Genomic sequence of the LATS2 gene needs to be performed to demonstrate the nature of the genetic inactivation of LATS2. Furthermore,

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Western blotting should be performed to show that LATS2 protein is no longer induced in high passage cells.

Authors Response: We thank the reviewer for the constructive suggestions.
As discussed above (Review#1, question 6), we used pooled primary cells to perform our experiments. When immortalized cell line is used in the experiment, cloning selection is necessary to get a pure cell cloning for the CRISPR/Cas9-based gene deletion. However, in our experiments, we use primary human cells. These primary cells will gradually become senescence during culture. For example, an apparent senescent phenotype is observed in hOSEs at their 13th passage. hOSE-YAPS127A cells become senescent at the 7th passage. Therefore, in tumorigenic studies, it was easy to collect enough LATS2-downregulated hOSE-YAPS127A cells (rapid growth, resistant to senescence), but rather difficult to collect enough control cells (hOSE cells and hOSE-YAPS127A
cells) with the similar conditions (cell density, number of passages and culturing time) for these studies.

To overcome this problem, we made a LATS2 knockout cell population using a LATS2 sgRNA CRISPR (Vector: pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro) All-in-One Lentivirus set (Applied Biological Materials Inc. Catalog# K1198315). The target sequences and a detailed knockout protocol have been added to the "Materials and Methods" section in the revised version of the manuscript. qRT-PCR and semi-quantitative PCR results showed that LATS2 mRNA was undetectable in the 7th passage cells, indicating that LATS2 was knocked out in the majority of hOSE cells. To further confirm our result, we stained LATS2 and CAS9 in a LATS2KO-YAPS127A
cell-derived tumor. As shown in Figure 8D, LATS2 immunosignal was detected only in the connective tissues, but not in the tumor cells. We also stained Cas9 in these cells and found that Cas9 was highly expressed in all tumor cells, but not in tumor-associated connect tissues (Appendix figure S13). These results demonstrated that LATS2 was knocked out in LATS2KOYAPS127A hOSE cells.

Since down-regulation of LATS2 induced cell proliferation and blocked senescence, LATS2 knockout hOSEs were enriched with culture and became the dominant cell population in the pooled cells, while LATS2 positive cells were eliminated by puromycin selection. Even if few cells escaped from the drug selection, they would become senescent and gradually wiped out by passaging (senescent cells have difficulty to re-attached to the culture dishes).

Reviewer's comment 2. In Figure 2, the authors claimed that hyper-activation of YAP induces cellular senescence in primary human cells. In contrast, the authors also stated "These data suggest that hOSEYAP and hOSE-YAP(S127A) cells were arrested in late G1 and S phases (Figure 2C)". Cell cycle arrest and cellular senescence are not same. The authors need to confirm what they observed in YAP-overexpressing cells is truly cellular senescence. More phenotypic characterization (in addition to SA-b-gal staining and WB analysis, such as SAHF formation) may further clarify if these cells are indeed senescent.

Authors Response: We thank the reviewer for the constructive suggestions. Senescence was classically defined as the state of permanent and irreversible cell cycle arrest. However, as mentioned by the reviewer, cell cycle arrest cannot be simply considered as senescence.

Although no single signature of the senescent phenotype is exclusively specific, hallmarks of senescent cells have been identified, including an essentially irreversible growth arrest, increase of cellular size, expression of SA-β-gal, up-regulation of senescence-associated secretory phenotype (SASP) factors and formation of senescence-associated heterochromatin foci (SAHF) (Francis Rodier and Judith Campisi, 2011). To further confirm the senescence of the late passage hOSE-YAP and hOSE-YAPS127A cells, we examined the expressions of several well-known SASP factors (FGF2, VEGFA, IL6, and IL8) and stained two SAHF makers (macroH2A and H3K9Me3) in these cells. As shown in Figure EV1, all examined SASP factors were significantly up-regulated in these cells. Moreover, macroH2A and H3K9Me3 also accumulated and formed foci in these cells (Figure EV2). These results, together with the featured cellular morphology and SA-β-gal

staining, convincingly indicate that hyperactivation of YAP1 induced senescence in primary hOSE cells.

Reviewer's comment 3. In all SA‑b‑gal staining data, the authors need to provide quantitative information.

***Authors Response**:* We thank the reviewer for her/his constructive questions. Quantitative results for all SA-β-gal staining were added to the manuscript (Fig. 2B; Fig. 3D & 3E; Fig. 5G; Fig. 6E; Fig. 7H & 7L. Appendix Figure S6, S9C, S10B).

Reviewer's comment 4. In Figure 4, the authors demonstrated that silencing of RB1 expression prevented YAP‑induced cellular senescence. The authors also showed that deletion of LATS2 prevented

YAP‑induced senescence (Figures 6E¬‑G). However, in contrast, overexpression of LATS2 induced cellular

senescence both in the presence or absence of RB1 (Figures 7J‑L). Those data are not easily reconciled

with the authors' model that YAP induces LATS2 expression to induce cellular senescent program (Figure

8). The authors need to provide some explanation or further experimentation to clarify this discrepancy.

Authors Response: We thank review for his insightful comments. As mentioned, in the primary ovarian surface epithelial (hOSE) cells, hyperactivation of YAP1 induces two critical tumor suppressors, LATS2 and RB1. Upregulated of RB1 and LATS2 induce cellular senescence to prevent cells from malignant transformation. Increased RB1 may interact with activating E2Fs (E2F1, E2F2, E2F3A) to suppress transcription of E2F-driven genes, leading to arrest cell cycle, which is an essential step for the establishment of cellular senescence. Our mechanistic studies also showed that increased LATS2 may drive the assembly of the DREAM complex. DREAM complex is known suppressor of E2F-driven genes and increased DREAM activity also lead to arrest of cell cycle and establishment of cellular senescence. Interestingly, we can see that RB1 and LATS2 signaling pathways converge in the regulation of E2F downstream genes. Although the role of RB1 in senescence has been well-documented, we found that LATS2 can induce cellular senescence regardless of RB1 expression, suggesting that a high level of LATS2 is a major player in senescence establishment.

Importantly, LATS2 is the known upstream suppressor of YAP activity in the Hippo signaling pathway. Up-regulation of LATS2 by hyper-activated YAP1 indicates that YAP1 and LATS2 form a negative feedback loop to prevent over-activation of YAP1 in normal cells. Although RB1 is also increased by hyperactivation of YAP1, silencing of LATS2 is sufficient to overcome YAP1-induced senescence in hOSEs. These data indicated that RB1 might serve as a redundant (but very important) manostat employed by normal hOSE cells to prevent themselves from YAP1-induced malignant transformation. To focus our study, in this manuscript, we mainly concentrated on
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revealing the role of YAP1-LATS2 feedback loop in the hOSE senescence and the mechanism(s) by which the YAP1-LATS2 feedback loop dictate ovarian cell fate (cellular senescence Vs. malignant transformation). As mentioned. RB1 is also an important player of YAP1 associated cellular senescence and tumorigenesis. This information has been added to the discussion of the revised version of the manuscript (This response letter will also be published per the policy of *EMBO Reports*).

Reviewer's comment 5. Figure 6A shows that LATS protein levels were not significantly increased in the

YAP overexpressing cells. This data contradicts to the statement in the text "As shown in Figure 6A, the

LATS protein was elevated in hOSE‑YAP and hOSE‑YAP S127A cells". Importantly, this data argue against

a model that LATS2 induction directly contributes to senescence. What is the authors' explanation?

Authors Response: We thank the reviewer for her/his question.
Both mRNA and Western blot assay showed LATS expression was significantly elevated by hyperactivation of YAP1 in hOSE cells. The reviewer did not identify the difference of LATS protein
in the immunoblot in Figure 6A probably because the membrane was stripped several times and the band is too weak and we did not adjust the contrast of the image. We re-did the experiment and replaced the previous representative blot in the revised version of the manuscript.
Reviewer's comment 6. *Figure 7A, more description is deeded for the LATS2 expressing cells. Is this a stable clone? The expression levels of the ectopic LATS2 should be shown and compared with the endogenous LATS2 level by Western. Are YAP phosphorylation and cytoplasmic localization increased in the LATS2 expressing cells? Figure 7F-H, ectopic expression of LATS2 had little effect on senescence at early passages, indicating that LATS induction is not sufficient to induce senescence. This data needs to be explained.*

Authors Response: We thank the reviewer for the insightful comments.
LATS2 expressing cells were selected with puromycin after transfection of primary human cells with lentivirus-based LATS2 expressing vectors. Therefore, these cells could be seen as a stable line. Although it is difficult to examine the expression of LATS2 protein in hOSE-LATS2 cells because of the availability of LATS2 antibody for Western blotting analysis, the transfection efficiency of LATS2 vector was verified via GFP expression under a fluorescent microscope (Figure 7A). As mentioned above, the LATS2 antibody in our laboratory is specific for IHC (verified
with knockout tissue). The IHC studies showed that LATS2 level was drastically increased in LATS2 overexpressed cells (Appendix Fig. S12). Moreover, the expression of LATS2 was confirmed by real-time-PCR, which indicated that LATS2 mRNA increased significantly in these cells (an approximately threefold increase compared to control). Most importantly, we found that compared to the control cells (hOSEs transfected with empty control vectors), hOSEs transfected with LATS2 vector stop growing (figure 7C, 7D, & 7E) and showing senescent phenotype (Figure 7G,7H, 7K & 7L, Appendix Fig. S12).

Our results showed that LATS2 plays critical roles in the establishment of natural replicative and YAP1-induced senescence. We agree with the reviewer that establishment of senescence is a complicated and relatively long process which involves activation or suppression of many different signaling pathways and reprogramming of many existing cellular biological events. This is reflected by the observation that ectopic expression of LATS2 had limited (but statistically significant, $P < 0.05$, compared to control) effect on SA-β-GAL positive cells in the early passage hOSEs. The slight but significant increase in SA-β-GAL positive cells in LATS2OV cells indicated that the senescent status was establishing in these cells [Figure 7F]. However, establishment of a complete senescent phenotype needs accumulation of senescence cells, which depends on the ratio of proliferative cells, apoptotic cells and cells with permanent cell cycle arrest (senescent). At passage 7 and passage 9, the majority of hOSEs are in senescence (Figure 7G & 7H). Moreover, LATS2 induction of senescence is cell type-dependent. As shown in Appendix figure S12, ectopic expression of LATS2 can induced senescence in the HUVEC cells within several days).

2nd Editorial Decision 4 October 2018

Thank you for the re-submission of your research manuscript to EMBO reports. We have now received reports from the same referees that have seen the first version of the paper, that were asked to re-evaluate your study (which can be found at the end of this email).

As you will see, referees #1 now supports the publication of the study in EMBO reports, whereas referee #3 asks for a minor revision. However, referee #2 has still major concerns, mainly regarding the lack of co-staining for melanocyte markers. We therefore ask you to add further experimental data to address the concerns of referee #2 in a final revised version of the manuscript. Please also add the Western blot data requested by referee #3 in his remaining point.

Further, I have already these editorial requests:

- Please upload the main figures the EV figures as editable TIFF or EPS-formatted single figure files in high resolution.

- We only allow up to 5 EV figures. Please arrange your data accordingly.

- Please provide the source data separated uploading one PDF file per figure.

- Please add a TOC with page numbers to the Appendix.

- Please remove any writing indicating the size from scale bars shown in the panels. Indicate the size only in the figure legends.

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When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures)
- the modified Appendix

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 about 400 pixels) that can be used as a visual synopsis on our website.

Please also note that we now mandate that all corresponding authors list an ORCID digital identifier that is linked to his/her EMBO reports account!

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

REFEREE REPORTS

Referee #1:

I have reviewed the revised manuscript for EMBOR-2017-44948V1, and believe that the authors addressed our concerns in a satisfactory manner.

Although the scope of this study is inherently limited in that the negative feedback regulation of YAP and LATS2 is not simply a unilateral mode of action and the simplicity in the experimental methodology taken by the authors, the general message and quality of data shown in this manuscript are now believed to be worthy of publication in EMBO Reports.

Referee #2:

My major concern was the fact that the authors stained LATS2 in skin and made claims about

expression levels changing in normal melanocytes versus transformed melanocytes, but without doing a co-stain for a melanocyte marker. We find that antibody stainings vary greatly from tumour to tumour and it is essential to normalise melanocyte protein expression against the keratinocytes. The author state that their antibody is not good enough for IF to allow them to do this essential experiment, but they don't show any data.

I still think that these stainings need to be done for the paper to be accepted. As it stands the authors have produced a lengthy rebuttal but have actually done very little to improve the core of their paper (almost all the figures are exactly the same, despite the many suggestions for improvements).

Referee #3:

The authors have addressed most of my concerns. On comment #1, the explanation for not doing DNA sequencing to verify LATS mutation is acceptable. However, it is very puzzling why the authors did not do a simple Western blot of LATS2 to show that LATS2 protein are indeed depleted for experiments in figure 5 and 6. I think that LATS2 Western blots need to be included to show that LATS2 protein is indeed eliminated in the LATS2 knockout cell pool.

2nd Revision - authors' response 27 November 2018

Point-by-point response to reviewer's concerns

Referee #1

Reviewer's comments: I have reviewed the revised manuscript for EMBOR-2017-44948V1, and believe that the authors addressed our concerns in a satisfactory manner.
Although the scope of this study is inherently limited in that the negative feedback regulation of YAP and LATS2 is not simply a unilateral mode of action and the simplicity in the experimental methodology taken by the authors, the general message and quality of data shown in this manuscript are now believed to be worthy of publication in EMBO Reports.

Author's response: We thank the reviewer for the time and professional comments. We believe that our future follow-up work and work of our peers in this research field will utilize this feedback loop to develop new strategies for effective prevention and better treatment of cancers.

Referee #2

Reviewer's comments: My major concern was the fact that the authors stained LATS2 in skin and made claims about expression levels changing in normal melanocytes versus transformed melanocytes, but without doing a co-stain for a melanocyte marker. We find that antibody stainings vary greatly from tumour to tumour and it is essential to normalize melanocyte protein expression against the keratinocytes. The author state that their antibody is not good enough for IF to allow them to do this essential experiment, but they don't show any data.

I still think that these staining need to be done for the paper to be accepted. As it stands the authors have produced a lengthy rebuttal but have actually done very little to improve the core of their paper.

Author's response: We appreciate the reviewer's time and constructive comments. We stained LATS2 in nevus tissues because melanocytes in nevus tissues are known senescent cells in human body. We think that if LATS2 is critical for senescence, as we have observed, it should express in the melanocytes of nevus tissue at a high level to maintain the senescent status and prevent the malignant transformation. We do not want to address the role of LATS2 in the melanoma or other skin cancers because that is beyond our research focus. To our knowledge, Dr. Camargo's laboratory at the Harvard Stem Cell Institute has an ongoing project focusing on the role of the Hippo pathway in skin cancers.

We thank the reviewer's constructive suggestion for co-staining. With the kind support from Dr. David Fisher and Dr. Anna Mandinova in the Cutaneous Biology Research Center at Massachusetts General Hospital, we collected six cases of normal human skin samples. We also purchased normal skin and nevus tissue microarray from US-Biomax. The staining was performed with a Vector

laboratory Duet ImmPRESS™ Double staining polymer IHC kit (Cat#MP-7724). Melanocytes were identified with a melanocyte biomarker antibody cocktail, including antibodies against MART-1, tyrosinase, and gp100. The biomarker was visualized by a blue-gray color. We did not use typical brown color in our staining because melanin shows as yellowish-brown particles in these tissues. Our staining results clearly showed that skin melanocytes have no (or very low if any) LATS2. However, the melanocytes in nevus tissues have high-level expression of LATS2, which perfectly colocalize with melanocyte biomarkers (figure RR1, see next page). These results have been added to the revised manuscript (supplemental Figure S16). The materials and methods, as well as the results sections of the manuscript has been revised accordingly.

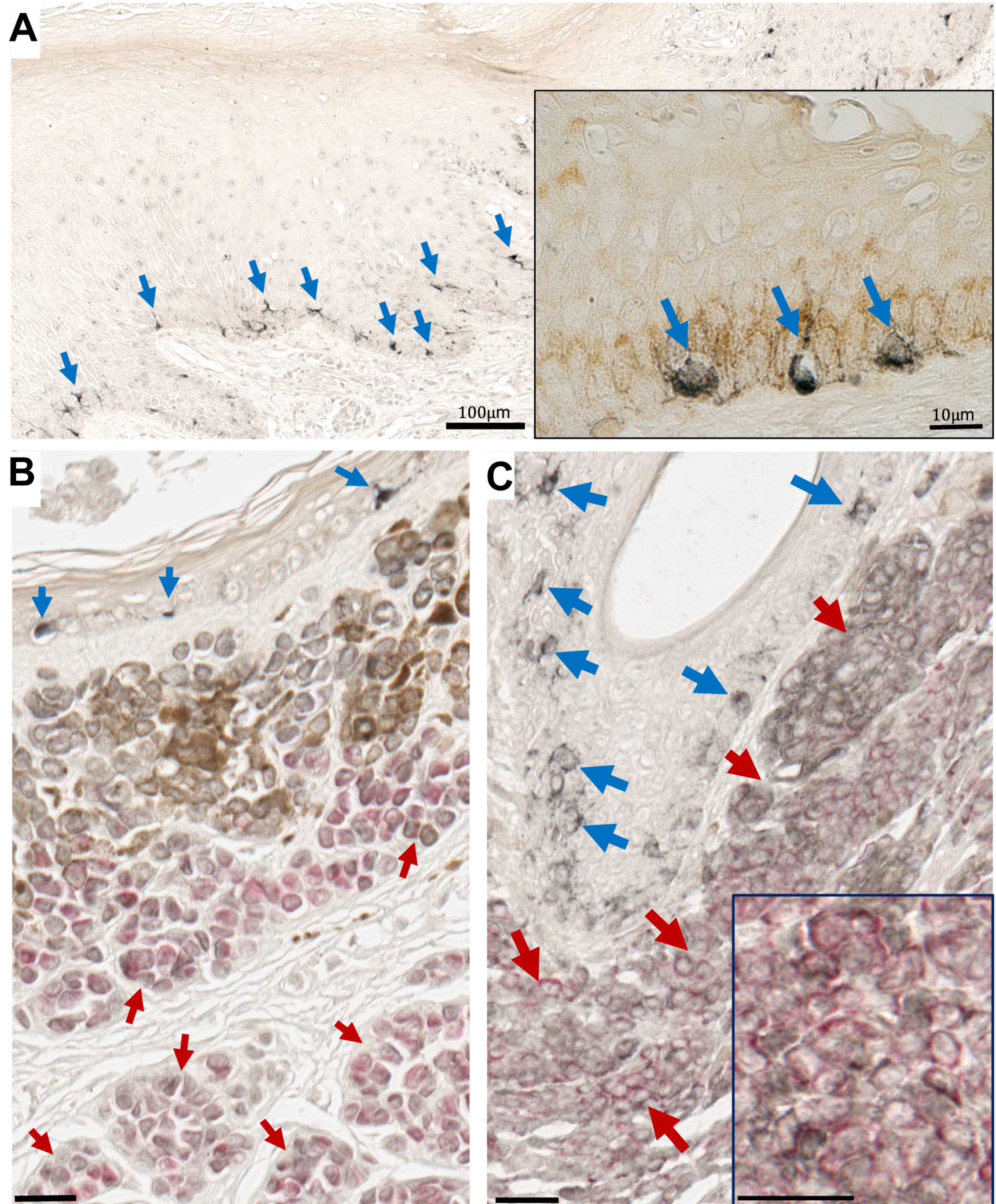

Figure RR1. Expression of LATS2 in melanocytes of nevus tissues. A) LATS2 (in red) is undetectable in the melanocytes. Melanocytes are cells with gray blue color, which are identified with a melanocyte biomarker antibody cocktail (MART-1, Tyrosinase, and gp100) using a dual color IHC staining kit, see detail in method section). Insert in (A) is a representative high-resolution image showing negative LATS2 staining in three melanocytes. The brown pigments are melanin. **B** & **C**) Two representative images showing the colocalization of LATS2 (in red) and melanocyte biomarker (in gray blue). LATS2 protein in skin melanocytes (blue arrows)

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is undetectable, but it is highly expressed in melanocytes of the Nevi tissue (red arrows). High resolution Insert in (C) showing the colocalization of LATS2 (red) and melanocyte biomarker (Gray) in Nevi. Scale bar: 25 μm.

Reviewer's additional comments: almost all the figures are exactly the same, despite the many suggestions for improvements.

Author's response: We appreciate reviewer's comments to improve our manuscript. In the past several months, we did many experiments to improve our manuscript before submission of the revised version. We addressed all concerns from other reviewers. Although we did not added new data in the main figures of our manuscript, we added new results in the supplementary figures (the number of supplementary figures increased from 9 to 14) and Expanded View figures (the number of EV figures increased from 3 to 7). Since *EMBO Reports* will published all reviewer's comments and response letters, data embedded in the rebuttal letter will also be published. Currently, we are generating transgenic mouse models to further support our findings.

Referee #3

Reviewer's comments*: The authors have addressed most of my concerns. On comment #1, the explanation for not doing DNA sequencing to verify LATS mutation is acceptable. However, it is very puzzling why the authors did not do a simple Western blot of LATS2 to show that LATS2 protein are indeed depleted for experiments in figure 5 and 6. I think that LATS2 Western blots need to be included to show that LATS2 protein is indeed eliminated in the LATS2 knockout cell pool.*

Author's response: We thank reviewer for the constructive suggestion. As mentioned, CRSPRY/Cas9 system functions in DNA levels. Theoretically, if no mRNA was detected, there will be no corresponding protein expression. We agree with the reviewer that a Western blot will provide stronger evidence for the elimination of LATS2 protein. <u>We did not present LATS2 western blot results because, to our knowledge, the LATS2-specific antibody for western blot is currently not available</u> (see Figure RR2 as an example of our previous Western blot antibody validation studies. A LATS2 antibody (#13646) used by several researchers has been discontinued by CST for some unknown reasons).

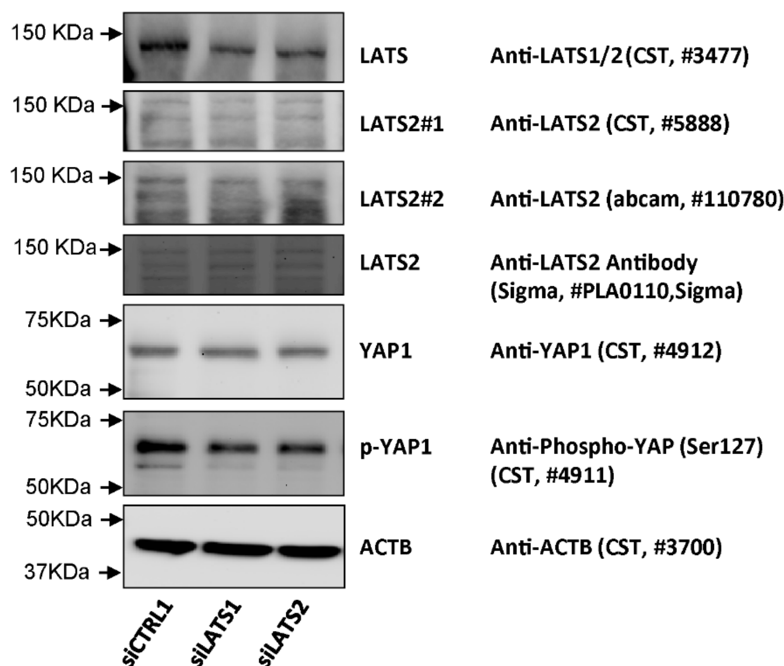

Figure RR2. Specificity of LATS2 antibodies for Western blot analysis. LATS1 and LATS2 were knocked down in hOSE cells with siRNAs (siLATS1 or siLATS2). Knockdown of LATS2 was confirmed by RT-PCR (see Fig. 5 and Fig. 6 in the article). Proteins in the same samples were loaded to the gel for western blot. Representative Western blotting images show that rabbit mAb LATS1 antibody (C66B5) (CST #3477) was able to detect both LATS1 and LATS2 in Western blot, while LATS2 antibodies from several major vendors, including the Cell Signaling Technology™ (CST), Abcam, and Sigma, did not detect any specific band in the same samples. YAP1 and phosphor-YAP1 were detected in the same membrane (as a positive control). Actin was used as protein loading control.

Actually, we also tried immunofluorescent (IF) staining with several available antibodies to detect LATS2 in frozen tissues and cells on coverslips. We observed cytoplasmic immunosignal that matched the expected cellular distribution of LATS2 in cultured cells (see Figure RR3 as an example). However, we consider these signals as false positive because this antibody also detects similar signal in some *LATS2* knockdown or knockout cells. To ensure the rigor of our data in this manuscript, we did not present any LATS2 protein results derived from the IF studies.

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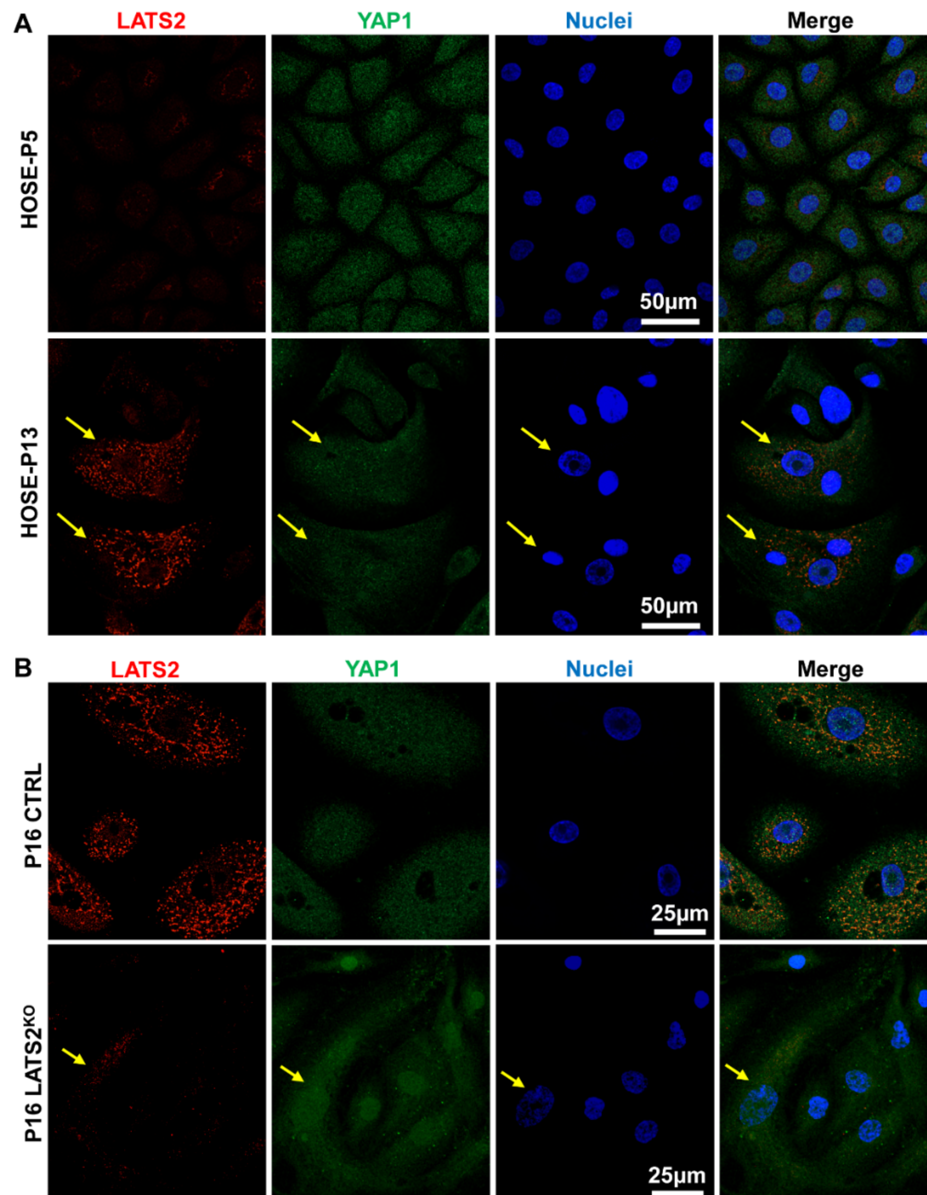

Figure RR3. Validation of LATS2 antibodies for fluorescent immunohistochemistry. LATS2 and YAP1 proteins in these cells were probed with antibodies against LATS2 (CST #5888) and YAP1 (CST #4912) in the fluorescent immunohistochemistry and visualized using Alexa 594 (red) and Alexa 488 (green)-conjugated second antibodies, respectively. Nuclei were stained with DAPI (blue). **A**) Representative images showing the expression of LATS2 (potentially) and YAP1 (confirmed) proteins in hOSE cells at their 5th passage (P5) or 13th passage (P13). **B**) Representative images showing the expression of LATS2 (potentially) and YAP1 (confirmed) in control and LATS2-knockout HUVEC cells at their 16th passage (P16). Scale bar: 50µm.

3rd Editorial Decision 13 December 2018

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. As you will see, both referees now support the publication of your manuscript in EMBO reports. Referee #2 has a final comment, but nevertheless indicates that we should proceed with acceptance.

Further, I have these editorial requests:

- Please provide the abstract written in present tense.

- It seems figures 7 and 9 are not called out in the text. Please add these callouts.

- The resolution of all Western blot panels at 100% is rather low. Please provide images with better resolution. See also:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

- Regarding the scale bars. Please provide all panels with microscopic images with scale bars. Please indicate the size of the bars in the respective figure legends (not within in the panels - do not write on the scale bars). There are still panels without scale bars (e.g. 3E, 7A, EV5A, S11 and S14), and panels with writing on the scale bars (e.g. S5, S7, S11).

- It seems author Bowen Ma is missing in the author contributions. Please provide this information.

- Please remove the conflict of interest statement and the author notes from the title page.

- Please format the references according to EMBO reports style. 'et al' should be used if there are more than ten authors. But the first 10 authors need to be shown! See:
http://embor.embopress.org/authorguide#referencesformat

- Please name the Table in the Appendix 'Appendix Table S1'. Please also add the word Appendix to the figure callouts in the title of the legends ('Appendix Figure Sx'). Please write the F of the word figure in the figure titles in capital, also in the TOC.

- Its seems the source data for Figure 2C has been labelled as source data for 2B. Please change this.

- The actin panel in 2C does not fit to the source data shown (4 lanes are marked in the SD, whereas the panel in the figure shows 3). Please fix this.

- The source data for Fig. 6A is incomplete, and does not fit to the panels shown in the figure. Please provide the correct and complete source data.

- Please also provide the source data for the Western blots shown in the Appendix.

- Please upload the source data files as ONE pdf-file per figure.

- Please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

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 final revision.

REFEREE REPORTS

Referee #2:

The manuscript is suitable for publication in EMBO reports without revision.

Referee #3:

Although I will not hold the paper for publication, the assertion that there is no good LATS2 antibody is incorrect. The authors might want to know that the CST LATS2 (D83D6) rabbit mAb #5888 antibody can detect endogenous LATS2 in many cell types.

3rd Revision - authors' response 22 December 2018

Referee #2:

The manuscript is suitable for publication in EMBO reports without revision.

Authors response: we really appreciate the reviewer for the constructive comments.

Referee #3:

Although I will not hold the paper for publication, the assertion that there is no good LATS2 antibody is incorrect. The authors might want to know that the CST LATS2 (D83D6) rabbit mAb #5888 antibody can detect endogenous LATS2 in many cell types.

Authors response: we appreciate the reviewer for agreeing to publish our results. We have CST LATS2 (D83D6) rabbit mAb (#5888) in the laboratory. We found that this antibody is more suitable for detecting LATS2 protein with immunohistochemistry.

4th Editorial Decision 8 January 2019

Thank you for the submission of your revised manuscript to our editorial offices. I now went through the manuscript files. There are still editorial requests that need to be addressed

- Please provide scale bars for Fig. 7A.

- Please name the Table in the Appendix 'Appendix Table S1'. The S is missing in the name. I cannot add this myself to a pdf file.

- The actin panel in 2C1 still does not fit to the source data shown. The first band in the source data shows a kink that is not present in the panel in the figue. Please fix this.

- Please add information on the data at P4 to the legend of Fig. EV2A.

- Please define the size of the scale bar in the legends of Fig. EV3A and Fig. EV5A.

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.

4th Revision - authors' response 22 December 2018

The authors performed all minor editorial changes.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND 🡻

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Cheng Wang
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-44948

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

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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)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For in vitro experiments, a minimum of three biological replicates (≥ 3 technical samples in each biological repeats) were included. This number is calculated by our preliminary studies based on an 80% power and a statistical significance level of 0.05.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For in vivo studies, we did not perform power analysis to determine the sample size because we knew that no tumor would be formed in the control groups. Sample size (10 tumors / group) was determined according to our previously published results. Mice were randomly distributed to each group before cell injection.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Because we focused on the tumorigenic ability of implanted cells, not tumor progression, all mice were euthalized 2 months after cell injection. No animals were excluded from the analysis.
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.	All mice were euthalized 2 months after cell injection, no treatment was performed on the control and tumor-carrying mice.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were picked for cell injection reandomly from cages.
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.	For in vitro experiments, cells were cultured in the dishes or culture paltes and the effects of subjective bias was minimal. For the in vivo study, due to randomisation of mice and no further treatment on these animals after cell injection, the potential effects of the subjective bias was really low.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Investigators were not blinded to the treatment groups because the tumor growth needed to be minitored every other day.
5. For every figure, are statistical tests justified as appropriate?	Yes. GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA) was used for statistic analysis. Data were analyzed for significance using student t-test (two groups) or one-way ANOVA with Tukey's post-hoc tests (multiple groups).
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Data were assumed to meet assumptions for ANOVA.The assumption was assessed according to our preliminary studies and published data.
Is there an estimate of variation within each group of data?	Yes. Since cultured cells were used in the in vitro experiments, the variation within each group will be very small. In most graphs, the mean & SEM (or SD) were presented. In some figures, box-and-whisker polts are used to display the median, interquartile and full ranges and outlying values.
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).	Antibody information, including vendor, catalog number/clone number, and the citation was provided in the Materials and Methods (and the Appendix table 1). Primers were purchased from the Real Time Primer, LLC (Elkins Park, PA). All primers were validated by the maufacture.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HUVEC cells were purchased from ATCC (Manassas, VA). hOSE, and HOMEC cells were purchased from ScienCell Research Laboratories (Carlsbad, CA). Human Ovarian Granulosa Cells were derived from the discarded granulosa cells IVF patients after oocyte retrieval. Cells from several patients were pooled before culture and treatment. Cell lines were authenticated using Short tandem repeat profiling performed by Genetica DNA laboratory (Burlington, NC).

* 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.	Female athymic nude mice (6 weeks of age) were purchased from the Charles River Laboratories (Wilmington, MA) and housed in a world-class animal facility with standard light/dark cycle and free access to food and water.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal handling and all experimental procedures were approved by the Institutional Animal Care and Use Committees (IACUC) of the University of Nebraska Medical Center and Masschusetts General Hospital.
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.	All animal procedures were reviewed and approved by UNMC IACUC and MGH IACUC. All investigators enrolled in this study had received occupational health and safety clearance, and were provided with necessary trainings in the humane care and use of laboratory animals, including federal policies/regulations governing the care and use of the laboratory animals, institutional policies and ethical principles governing animal care and use, research and testing methods that minimize animal pain and distress, non-animal alternatives, education resources, principles of anesthesia/pain monitoring, pre- and post-operative care.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not Applicable
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.	Not Applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not Applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not Applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable
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.	Not Applicable
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.	Not Applicable

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	Not Applicable
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).	Not Applicable
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).	Not Applicable
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.	Not Applicable

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.	Not applicable