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Mechanical cue-induced YAP instructs Skp2-dependent cell cycle exit and oncogenic signaling

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Transaction Report:

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

1st Editorial Decision 30 January 2017

Thank you for the submission of your manuscript (EMBOJ-2016-95679) to The EMBO Journal, and your patience with our response at this time of the year. My apologies again for the delay in processing the manuscript at this time of the year, which was due to delayed feedback from one of the referees, we however needed for our assessment. Your study has been sent to three referees, and we have received reports from all of them, which I copy below.

As you will see, the referees acknowledge the potential high interest and novelty of your work, although they also express a number of major concerns that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In more detail, referee #3 states that some of the claims related to Skp2 knockdown are not sufficiently supported by the data, and that in particular the epistasis experiments are not convincing in his/her view. Referee #1 asks you to extend your experiments to consolidate your findings that the Yap-Skp2 axis is not active in mouse cells. In addition, this referee states the need for you to confirm your results in the different cell lines utilized. Referee #1 agrees that the non-conserved phenotype of the signaling in mouse needs additional consideration, and that you should corroborate your analysis of the cancer tissue data. These issues, together with a number of additional technical requests and controls, raised by the other referees, need to be carefully addressed and clearly responded to in the point-by-point response.

I judge the comments of the referees to be generally reasonable and we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments.

REFEREE REPORTS

Referee #1:

Yang et al. report that mechanical cues trigger YAP activation and binding of YAP/TEAD to a TEAD binding consensus site in the Skp2 promoter. Consequently, Skp2 transcription becomes increased and p21 and p27 levels decreased. They went on to show that Skp2 is the major regulator of YAP depletion-induced cell cycle exit at G0. In several model systems (tumor spheroid-like acini formation, mammospheres) they confirmed the YAP-Skp2 signalling axis and showed a significant positive correlation of YAP and Skp2 expression in breast cancer patients. Interestingly, they also show convincing evidence that this signalling pathway is not conserved in mice. Hence, the suppression of oncogene-induced mammary tumours upon MMTV-Cre depletion of YAP in mice should therefore be mediated by a Skp2-independent mechanism. It will be important to uncover this mechanism and test its relevance in human cancer.
The manuscript reports novel findings that are interesting to the readership of EMBO J. The assays are well conducted (although some Western blots could be improved, eg. Figs 3E, 5A, EV2A, EV2). Furthermore, the usage of different cell lines for different assays is problematic. It would have been better to perform all assays at least with one cell line and conform distinct results with additional cel lines.

Referee #2:

Jang et al., investigate Skp2 F-box protein regulation by mechanical cues. They report that Skp2 mRNA levels are subject to regulation by cell detachment stimuli and implicate the transcriptional regulator and Hippo effector Yap in this process. They show that Yap can bind to endogenous TEAD binding sites in the Skp2 gene by ChIP and to activate transcription of a Skp2 promoter-reporter. In a series of experiments in which either activated Yap is overexpressed or endogenous Yap is depleted in combination with Skp2 modulation by various means (shRNAs, sgRNA targeting), the authors conclude that first, Skp2 can overcome cell cycle exit induced by Yap depletion, that second, Skp2 is not required for activated Yap-driven cell cycle exit and that third, Yap-driven tumor spheroid-like acini formation requires Skp2 function. Analysis of various microarray datasets reveals that increased expression of Skp2 correlates with Yap expression. Similar trends are observed from IHC stainings of breast cancer tissue.

The majority of data presented in this paper relate to phenotypes created by ectopic expression of activated Yap in the absence of presence of Skp2. Thus, the paper addresses what phenomena Yap can induce when overexpressed but does not directly address mechanical cue regulation of Skp2 by Yap. Also, data are shown demonstrating that Skp2 is not required for Yap-driven cell cycle exit. This is an isolated observation, which is not connected to mechanical cue regulation of Skp2 at the transcriptional level. Moreover, it remains unclear whether Yap indeed acts to control Skp2 mRNA expression in response to mechanical cues in settings of endogenous levels (particular evident in Fig. 2). Various mechanical stimuli and detachment should be employed and Yap binding to the Skp2 promoter studied by ChIP at the endogenous levels. The authors imply also that the regulation of Skp2 by Yap is a critical path used by mechanical cues to control the cell cycle. If so, it is surprising that this regulatory path is not conserved in mouse cells. This requires additional considerations. The analysis of Skp2/Yap expression in human breast cancer needs a more thorough statistical evaluation and correlation to Yap target gene expression. The reviewer recommends the authors to use the cBioPortal.org webpage to complement their analysis of this relationship in breast and other cancers. Finally, the reviewer notes that Yap function has been previously linked to the expression of cycle regulatory proteins including cyclin D1, E2F1 and cyclin E.

Referee #3:

In the manuscript of Jang, et al, the authors identified the cell cycle regulator and F-box protein Skp2 as a target gene of the Hippo pathway effector YAP and demonstrated that Skp2 is required for effects of activated YAP. Mechanistically, the authors propose that mechanical cues regulate YAP activity and Skp2 expression and that Skp2 expression is sufficient to counteract the senescence induced by p21/p27 when YAP is downregulated. Using a 3D culture system, they show that Skp2

inactivation suppresses YAP driven oncogenic behavior of cells.

Most of the findings of this paper are well supported by experimental data and provide interesting mechanistic insights into the mechanoregulation of cell cycle exit. However, a number of experiments are overinterpreted and this needs to be corrected.

Major concerns:
1. The epistasis with Skp2 knockdown is not informative because the Skp2 knockdown has only a very weak phenotype by itself. Thus YAP induced upregulation of Skp2 can simply rescue these weak defects by supplying a bit more Skp2, even though Skp2 may act downstream of YAP. Unless the authors find a way to show more dramatic phenotypes or use a null cell line for Skp2, this data is thus meaningless and needs to be removed. Otherwise the text is highly misleading.

2. In Figure 5E, the authors show that Skp2 knockdown induces lumen formation and restores the defect caused by YAP5SA. There is no explanation for this result. Is this because Skp2 restores polarity defects caused by YAP overexpression? What is the explanation for this and how does it fit with this story?

3. Although the authors suggest that Skp2 knockdown decreases phenotypes induces by YAP on stiff substrate for acini in Figure 5H (for both control and YAP5SA), those acini still look like malignant and invasive. Similarly, in EV3E, Skp2 inhibition decreases the phenotype induces by YAP5SA but those still look malignant. This needs to be mentioned in the text and discussed. In its current form the text is misleading as to the phenotype of the Skp2 knockdown.

Minor concerns:
1. Language should be corrected for publication.

2. Although, the study mainly depends on breast cancer cells, in some figures a retinal cell line is used. Please explain in the text why.

3. In several figures, the controls have no error bars. Is it because there is one replicate? Presumably the control experiments were also done in triplicate and error bars thus need to be added to the graphs.

4. In figure 2E, YAP binding peaks are present for both TEAD binding sites while authors suggest that YAP only binds to TB2. Please explain and correct.

5. Figure EV1E presents a TAZ western blot but only for the siControl. Why?

1st Revision - authors' response 24 April 2017

Referee #1:

Yang et al. report that mechanical cues trigger YAP activation and binding of YAP/TEAD to a TEAD binding consensus site in the Skp2 promoter. Consequently, Skp2 transcription becomes increased and p21 and p27 levels decreased. They went on to show that Skp2 is the major regulator of YAP depletion-induced cell cycle exit at G0. In several model systems (tumor spheroid-like acini formation, mammospheres) they confirmed the YAP-Skp2 signalling axis and showed a significant positive correlation of YAP and Skp2 expression in breast cancer patients. Interestingly, they also show convincing evidence that this signalling pathway is not conserved in mice. Hence, the suppression of oncogene-induced mammary tumours upon MMTV-Cre depletion of YAP in mice should therefore be mediated by a Skp2-independent mechanism. It will be important to uncover this mechanism and test its relevance in human cancer.

➔ Thank you for the suggestion. Because mice have been so commonly used as model organisms for understanding not only Hippo signaling, but also human physiology in general, it is very important to recognize any biological discrepancies between the two species. This will make it easier to identify the correct therapeutic approaches for human diseases including cancer. Thus, we fully agree we should further investigate whether the YAP-Skp2 axis is conserved in mice.

We agree that using MMTV-Cre for YAP knockout in oncogene-induced mammary tumours to see the relevance of Skp2 in this model is a good idea. To address Reviewer 1's request for more in vivo data, we would have to generate mice that develop oncogene-induced mammary tumours plus the MMTV-Cre; YAP f/f, Skp2 f/f genotypes. This would take far longer than the limited time we received for this revision. We, therefore, decided to take another, faster approach.

We have conducted new experiments in mouse mammary cell lines to further support our conclusions. Using siRNAs against mouse Yap, we have performed knock-down experiments in 4T1 and NMuMG cell lines. To our surprise, we found Yap depletion for 2 days decreases Skp2 protein levels (Fig 7C), similar to what we observed in human cell lines. We found, however, that this was likely an indirect consequence of cell cycle enrichment at G0/G1 induced by the Yap knock-down. This was reflected by a decrease in Cyclin B1, a G2/M cell cycle marker, in Yap-depleted cells (Fig 7C). Indeed, unlike in human cell lines (Fig 1G and Fig 7D, left), Yap-depleted 4T1 mitotic cells collected as in Fig 1G show similar Skp2 levels to knock-down control and mock mitotic control cells (Fig 7D, right). As expected, these cells do not show similar Ctgf levels. Importantly, MG132-induced inhibition of proteasomal degradation in Yap-depleted 4T1 cells rescues Skp2 protein levels (Fig 7E). This suggests the APC-Cdh1-mediated proteasomal degradation that usually occurs in early G1 phase may be responsible for the reduction in Skp2 levels observed in Yap-depleted 4T1 mouse cells. Of note, MG132 treatment does not rescue the reduction in Skp2 induced by YAP knock-down in human cell lines (Fig 1F and Fig EV1A).

To further confirm that Yap activation does not increase Skp2 mRNA levels in mice, we overexpressed YAP in 4T1 and NMuMG cells. We also used Lats1/2$^{-/-}$ MEFs. After validating the specificity of the Skp2 antibody for mouse Skp2 by looking at the Skp2 knock-down condition, we found Skp2 does not respond to Yap activation (Fig 7A, right and Fig 7B) in these cells either. We then analyzed microarray data generated from transgenic YAP mouse livers (Dong, Feldmann et al., 2007), but we did not find Skp2 among the list of genes up-regulated by YAP (data not shown).

Humans and mice are similar in many ways, but they also show obvious differences. It is not unexpected that some cellular signaling pathways will have diverged since the last common ancestor shared by mice and humans. This discussion and these new data have been added to the revised manuscript (page 14, line 10-page 15, line 13; page 17, line 9-13).

The manuscript reports novel findings that are interesting to the readership of EMBO J. The assays are well conducted (although some Western blots could be improved, eg. Figs 3E, 5A, EV2A, EV2).

➔ We appreciate these positive comments. As requested, we have improved the quality of the Western blots. (Figs. 3E → new p21 blot with a shorter exposure. / Fig. 5A, Fig. EV2A, Fig. EV2D → We repeated this experiment and have included new blot images.)

Furthermore, the usage of different cell lines for different assays is problematic. It would have been better to perform all assays at least with one cell line and conform distinct results with additional cell lines.

➔ Thank you for this suggestion. As requested, we repeated in MCF10A cells most of the assays originally performed only in RPE1 or MDA-MB231 cells. Since we obtained results consistent with our original results, we are confident in our conclusions. The new data appear in Fig 1A, right / Fig 1E, middle / Fig 1H, right / Fig 7D, left / Fig EV1A and 1D, below.

We thank the reviewer for all of his/her constructive and helpful suggestions.

Reviewer #2:

Jang et al., investigate Skp2 F-box protein regulation by mechanical cues. They report that Skp2 mRNA levels are subject to regulation by cell detachment stimuli and implicate the transcriptional regulator and Hippo effector Yap in this process. They show that Yap can bind to endogenous TEAD binding sites in the Skp2 gene by ChIP and to activate transcription of a Skp2 promoter-reporter. In a series of experiments in which either activated Yap is overexpressed or endogenous Yap is

depleted in combination with Skp2 modulation by various means (shRNAs, sgRNA targeting), the authors conclude that first, Skp2 can overcome cell cycle exit induced by Yap depletion, that second, Skp2 is not required for activated Yap-driven cell cycle exit and that third, Yap-driven tumor spheroid-like acini formation requires Skp2 function. Analysis of various microarray datasets reveals that increased expression of Skp2 correlates with Yap expression. Similar trends are observed from IHC stainings of breast cancer tissue.

1) The majority of data presented in this paper relate to phenotypes created by ectopic expression of activated Yap in the absence of presence of Skp2. Thus, the paper addresses what phenomena Yap can induce when overexpressed but does not directly address mechanical cue regulation of Skp2 by Yap. Also, data are shown demonstrating that Skp2 is not required for Yap-driven cell cycle exit. This is an isolated observation, which is not connected to mechanical cue regulation of Skp2 at the transcriptional level. Moreover, it remains unclear whether Yap indeed acts to control Skp2 mRNA expression in response to mechanical cues in settings of endogenous levels (particular evident in Fig. 2). Various mechanical stimuli and detachment should be employed and Yap binding to the Skp2 promoter studied by ChIP at the endogenous levels.

➔ Thank you for this insightful suggestion. We have added the endogenous YAP ChIP-qPCR data from MCF10A cells subjected to various mechanical stimuli such as cellular suspension, inhibition of the actin cytoskeleton, or culturing in highly dense monolayers. Consistent with the changes we observed in Skp2 mRNA levels (Fig 2I, K), various mechanical stresses reduce the binding of endogenous YAP to the Skp2 promoter TB2 site (Fig 2L, M and Fig EV1I). Together, these data support a role for YAP in directly mediating mechanical cue-dependent Skp2 transcription.

➔ Here, we would like to respectfully point out a misinterpretation in Reviewer 2's summary of our study. Review 2 said, *"Also, data are shown demonstrating that Skp2 is* ***not*** *required for Yap-driven cell cycle exit"*.
We believe the word "not" was inserted by mistake as our entire manuscript is dedicated to showing that Skp2 is required for Yap-driven cell cycle exit.

2) The authors imply also that the regulation of Skp2 by Yap is a critical path used by mechanical cues to control the cell cycle. If so, it is surprising that this regulatory path is not conserved in mouse cells. This requires additional considerations.

➔ We appreciate Reviewer 2's thoughtful comments and suggestions. We fully agree that our hypothesis that the YAP-Skp2 axis is not conserved in mice needs further evaluation. We have conducted new experiments in mouse mammary cell lines to further support our conclusions. Using siRNAs against mouse Yap, we have performed knock-down experiments in 4T1 and NMuMG cell lines. To our surprise, we found that Yap depletion for 2 days decreases Skp2 protein levels (Fig 7C), similar to what we observed in human cell lines. We found, however, that this was likely an indirect consequence of cell cycle enrichment at G0/G1 induced by the Yap knock-down. This was reflected by a decrease in Cyclin B1, a G2/M cell cycle marker, in Yap-depleted cells (Fig 7C). Indeed, unlike in human cell lines (Fig 1G and Fig 7D, left), Yap-depleted 4T1 mitotic cells collected as in Fig 1G show similar Skp2 levels to knock-down control and mock mitotic control cells (Fig 7D, right). As expected, these cells do not show similar Ctgf levels. Importantly, MG132-induced inhibition of proteasomal degradation in Yap-depleted 4T1 cells rescues Skp2 protein levels (Fig 7E). This suggests the APC-Cdh1-mediated proteasomal degradation that usually occurs in early G1 phase may be responsible for the reduction in Skp2 levels observed in Yap-depleted 4T1 mouse cells. Of note, MG132 treatment does not rescue the reduction in Skp2 induced by YAP knock-down in human cell lines (Fig 1F and Fig EV1A).

To further confirm that Yap activation does not increase Skp2 mRNA levels in mice, we overexpressed YAP in 4T1 and NMuMG cells. We also used Lats1/2$^{-/-}$ MEFs. After validating the specificity of the Skp2 antibody for mouse Skp2 by looking at the Skp2 knock-down condition, we found Skp2 does not respond to Yap activation (Fig 7A, right and Fig 7B) in these cells either. We then analyzed microarray data generated from transgenic YAP mouse livers (Dong et al., 2007), but we did not find Skp2 among the list of genes up-regulated by YAP (data not shown).

Humans and mice are similar in many ways, but they also show obvious differences. It is not unexpected that some cellular signaling pathways will have diverged since the last common ancestor

shared by mice and humans. This discussion and these new data have been added to the revised manuscript (page 14, line 10-page 15, line 13; page 17, line 9-13).

3) The analysis of Skp2/Yap expression in human breast cancer needs a more thorough statistical evaluation and correlation to Yap target gene expression. The reviewer recommends the authors to use the cBioPortal.org webpage to complement their analysis of this relationship in breast and other cancers.

➔ Thank you for the helpful comments. As suggested, we have confirmed the correlation between YAP and Skp2 expression in cancers from various tissues including breast using the cBioPortal.org webpage. Consistent with our results, we found a statistically significant positive correlation between YAP and Skp2 mRNA (Fig EV5B). We then compared the expression of Skp2 and YAP target gene sets (downloaded from Cordenonsi_YAP_conserved_signature) in breast and other cancer patients. Although the correlation was imperfect, even among YAP signature genes, we did observe a tendency among cancer patients who had upregulated Skp2 to also show expression of Yap signature genes (Fig 8B and Fig EV5C).

4) Finally, the reviewer notes that Yap function has been previously linked to the expression of cycle regulatory proteins including cyclin D1, E2F1 and cyclin E.

➔We have added this to the discussion section of the revised manuscript (page 17, line 14-16).

We thank Reviewer 2 for all of his/her constructive and helpful suggestions.

Reviewer #3:

In the manuscript of Jang, et al, the authors identified the cell cycle regulator and F-box protein Skp2 as a target gene of the Hippo pathway effector YAP and demonstrated that Skp2 is required for effects of activated YAP. Mechanistically, the authors propose that mechanical cues regulate YAP activity and Skp2 expression and that Skp2 expression is sufficient to counteract the senescence induced by p21/p27 when YAP is downregulated. Using a 3D culture system, they show that Skp2 inactivation suppresses YAP driven oncogenic behavior of cells.

Most of the findings of this paper are well supported by experimental data and provide interesting mechanistic insights into the mechanoregulation of cell cycle exit. However, a number of experiments are overinterpreted and this needs to be corrected.

Major concerns:

1) The epistasis with Skp2 knockdown is not informative because the Skp2 knockdown has only a very weak phenotype by itself. Thus YAP induced upregulation of Skp2 can simply rescue these weak defects by supplying a bit more Skp2, even though Skp2 may act downstream of YAP. Unless the authors find a way to show more dramatic phenotypes or use a null cell line for Skp2, this data is thus meaningless and needs to be removed. Otherwise the text is highly misleading.

➔ It is not entirely clear to what Reviewer 3 is referencing when saying, "*this data is thus meaningless and needs to be removed"*.

It seems Reviewer 3 interpreted Fig. 4 in a way other than what we intended. We agree that most of the data in Fig. 4 does not support epistasis between Skp2 and YAP. In Fig. 4 and Fig EV2 A-F, we showed that neither knockdown nor knockout of Skp2 is effective in suppressing YAP-driven cell proliferation or cell cycle re-entry in "2D" culture. We did clearly mention, however, that, rather than arguing for functional epistasis between Skp2 and YAP, Fig. 4 indicates *"YAP hyper-activation can bypass cell cycle exit in the absence of Skp2 in a 2D culture system*." In fact, we showed Skp2 depletion represses the formation of YAP-driven tumor spheroid-like acini and aberrant epithelial tissue behaviors in 3D stiff matrix (Fig 5). Thus, it is Figure 5 that indicates functional epistasis of Skp2 and YAP in 3D culture. By showing Fig. 4 and Fig. 5 together, we hope our readers will understand that specific cellular phenotypes of 5SA-YAP expressing cells grown in 2D versus 3D conditions differ upon Skp2 inhibition. Thus, we respectfully disagree with removing Fig. 4. Rather

we request reconsideration of the fact that we did not intend to claim that YAP and Skp2 exhibit functional epistasis from the data in Fig. 4 alone. Instead, to avoid confusion, we have revised the title of Fig. 4 to now read, "**In a 2D culture system, Skp2 inhibition does not effectively suppress YAP-driven cell proliferation or cell cycle re-entry**."

➔ When Reviewer 3 refers to "this data," he or she may also be referring to the 3D acini size data in Fig 5B-D. It may be confusing why Skp2 inactivation in control cells does not reduce the size of 3D acini compared to shCTL or DMSO-treated cells. "Soft" tissue microenvironments that restrict cell overgrowth are thought to facilitate tissue homeostasis (Paszek, Zahir et al., 2005). This usually leads to acinar growth arrest within 7 days. This phenomenon does not seem, however, to depend solely on YAP inactivation, as we have observed nuclear YAP localization even in soft 3D tissue cultures (Fig EV4A). Skp2-depleted cells (achieved either via shRNA-mediated knock-down or knockout by CRISPR) still proliferate, albeit slowly (Fig EV2B, F). Similarly, Skp2 knockout mice show normal development despite having smaller body size than wild type (Nakayama, Nagahama et al., 2000). For this reason, we think the 3D soft tissue microenvironment itself is the dominant factor in the growth arrest of control acini because it overrides the effect of Skp2 inactivation. We believe, however, that YAP hyper-activation can overcome this 3D tissue-dependent growth arrest and that Skp2 induction is required for this to occur. Finally, we note that Skp2 depletion suppresses YAP-driven irregular 3D acinar growth, but not the growth of control acini. This implies Skp2 may prove to be a reliable therapeutic target for human cancers that exhibit YAP hyper-activation.

We hope this explanation satisfies Reviewer 3.

2) In Figure 5E, the authors show that Skp2 knockdown induces lumen formation and restores the defect caused by YAP5SA. There is no explanation for this result. Is this because Skp2 restores polarity defects caused by YAP overexpression? What is the explanation for this and how does it fit with this story?

➔ This is a critical question, but to answer it, we realized we needed to understand how lumen formation in 3D mammary acini works. Dr. Joan Brugge's group from Harvard University has long studied the development of 3D acini as a model for mammary ductal formation *in vivo*. According to one of their studies (Schafer, Grassian et al., 2009), inner cells of 3D acini lose attachment from the ECM and exhibit a "glucose uptake defect" via impaired Akt signaling. This leads to the death of inner acinar cells, leaving behind a hollow lumen. Interestingly, two recent papers have suggested Skp2 can promote glucose uptake and aerobic glycolysis via Akt regulation (Chan, Li et al., 2012, Chan, Morrow et al., 2013).

With this in mind, we first tested whether 5SA-YAP-expressing cells that detach from the ECM can maintain glucose uptake. We measured glucose uptake after seeding the same number of cells (6 X 10^4/200 µl) across poly-HEMA-coated 96-well plates and keeping them suspended for 20 hrs. Consistent with the Brugge group's study, we observed reduced glucose uptake in control cells detached from the ECM. 5SA-YAP expressing cells, in contrast, show relatively high levels of glucose uptake compared to control cells regardless of whether they grown in adherent conditions or in suspension (Fig 5G). We also found that the media of both adherent and suspended 5SA-YAP-expressing cells became yellowish, suggesting YAP hyper-activation accelerates the production of acidic metabolites, perhaps through aerobic glycolysis (Fig 5G). Consistent with our results, Dr. Junjie Chen's group reported similar observations despite only testing the cellular adhesion condition (Wang, Xiao et al., 2015).

We next asked whether the high glucose uptake maintained during ECM detachment in 5SA-YAP-expressing cells depends on the increased levels of Skp2. We found Skp2 depletion effectively down-regulates glucose uptake in suspended 5SA-YAP-expressing cells (Fig 5H). We also found Skp2 depletion reduces the production of the aerobic glycolysis by-product lactate (Fig 5I).

Consistent with the report that Skp2 controls glucose uptake and glycolysis via Akt, we found 5SA-YAP-expressing cells treated with the PI3K inhibitor (LY294002) show reduced glucose uptake and lactate production (Fig EV3A, B). Glucose uptake may reflect an increase in glucose transporter expression. Interestingly, Wang et al. suggested 5SA-YAP induces glut3 expression and Chan et al. suggested the Skp2-mediated changes in glucose uptake are the result of Akt-induced changes in glut1 expression (Chan et al., 2012). Thus, we measured glut1 and glut3 expression in the same

experimental settings by qRT-PCR. Consistent with previous reports, we found 5SA-YAP overexpression increases glut3 expression, but causes no further enhancement of glut1 expression. Skp2 depletion, in contrast, has no effect on glut3 expression, but reduces glut1 mRNA expression (Fig EV3C). These data imply that during ECM detachment, 5SA-YAP expressing cells may be able to maintain high levels of glucose uptake via glut1 expression which is sustained by enhanced Skp2 as well as increased glut3.

Given that inner cell detachment from the ECM is accepted as the major developmental mechanism driving 3D mammary lumen formation, the data we provide here, mostly using cellular suspensions, seem to suggest that although 5SA-YAP-expressing cells fail lumen formation, Skp2 knock-down can partially rescue it. These data and discussion now appear in the revised manuscript (page 10, line 12 - page 12, line 5).

3) Although the authors suggest that Skp2 knockdown decreases phenotypes induces by YAP on stiff substrate for acini in Figure 5H (for both control and YAP5SA), those acini still look like malignant and invasive. Similarly, in EV3E, Skp2 inhibition decreases the phenotype induces by YAP5SA but those still look malignant. This needs to be mentioned in the text and discussed. In its current form the text is misleading as to the phenotype of the Skp2 knockdown.

➔ We fully agree and thank you for addressing this critical concern. Indeed, we found that although 5SA-YAP overexpression substantially increases levels of the mesenchymal marker N-cadherin, Skp2 knock-down does not affect this phenotype (Fig 5A). Thus, it seems the YAP-driven increase in Skp2 does not likely to contribute to the invasive EMT phenotype induced by 5SA-YAP expression in cells on stiff 3D substrates. We have added a discussion of this point to the revised manuscript and removed the word "malignant" to avoid any misunderstanding.

Minor concerns:
1. Language should be corrected for publication.
➔ We have revised the manuscript and had it edited by a professional service.

2. Although, the study mainly depends on breast cancer cells, in some figures a retinal cell line is used. Please explain in the text why.
➔ The trivial reason is that we began this project using RPE1 cells. Later, we moved to breast cell lines because so many studies on Hippo signaling were done in mammary cells. We performed the same experiments on breast cell lines and obtained the same results. We have now added the new data to the revised manuscript. (newly appended data: Fig 1E, middle / Fig 1H, right / Fig 7D, left).

3. In several figures, the controls have no error bars. Is it because there is one replicate? Presumably the control experiments were also done in triplicate and error bars thus need to be added to the graphs.
➔ All control experiments also had multiple replicates. In the qRT-PCR experiments, the average value obtained from technical replicates of the control experiment was set as "1" to normalize the other experimental values. Thus, although we conducted biological replicate experiments, control was identically set to "1". This is why the controls in the qRT-PCR data have no error bars. This is fairly common practice as the following papers also lack error bars on their controls in experiments using normalization.

1. Yi Tang et al. 2016. *Nat. Cell Biol*. Snail/Slug binding interactions with YAP/TAZ control skeletal stem cell self-renewal and differentiation.
2. Liu et al. 2016. *Cell.* Toll Receptor-Mediated Hippo Signaling Controls Innate Immunity in Drosophila.

4. In figure 2E, YAP binding peaks are present for both TEAD binding sites while authors suggest that YAP only binds to TB2. Please explain and correct.

➔ Thank you for your comment. We were not trying to argue that YAP binds only to TB2 and not TB1. Instead, we focused only on the TB2 site. As we described, our ChIP-seq experiment was conducted on 5SA-YAP overexpressing cells. The ChIP-seq experiments of Stein et al. were done at endogenous levels of YAP and TEAD1. Only the TB2 site appeared in both our results and the results of the Stein group. Since we assumed that the endogenous-ChIP-seq results would be more reliable than those in the overexpression condition, we chose to analyze only the TB2 peak. We

cannot, however, rule out the possibility that TB1 also participates in YAP-mediated Skp2 expression.

5. Figure EV1E presents a TAZ western blot but only for the siControl. Why?

➔ In the revised manuscript, Figure EV1F is now Figure EV1E. We used TAZ as a positive control for MG132 treatment because TAZ protein has a very short half-life. The trivial explanation for this is, at that time, we did not have enough TEAD knock-down sample lysates for the TAZ immunoblot. To avoid unwanted confusion, we now use c-Myc instead of TAZ as a positive control for MG132 for the same experiment (Fig EV1F).

We thank the reviewer for all of his/her constructive and helpful suggestions.

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2nd Editorial Decision 01 June 2017

Thank you for submitting the revised version of your manuscript. I apologise for the delay in getting back to you, which is due to delayed feedback from one of the referees. Your revised manuscript has now been seen by two of the original referees, whose comments are enclosed below. Please note that while the second referee could not look back at the revised work this time, we have carefully assessed your point-by-point response to his/her concerns as well.

As you will see, both referee #1 and referee #3 find that their concerns have been sufficiently addressed and are now broadly in favour of publication. In addition, we conclude that the concerns of referee #2 have also been adequately addressed in the revisions.

Thus, I am pleased to inform you that we are happy to accept your manuscript for publication in The EMBO Journal, pending satisfactory revision of a few editorial issues concerning text and figures that I need you to address.
I look forward to your final revision.

REFEREE REPORTS

Referee #1:

The revisions are extensive and clarified the suggestions and concerns I raised in my initial review. This paper reports interesting findings and should be published in EMBO J.

Referee #3:

My concerns were addressed.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dae-Sik Lim
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-96089

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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 $\chi 2$ 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

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

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

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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

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

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

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

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).	We have described all the relevant informations in Materials and Methods section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All the cell lines used in this study have been tested for mycoplasma contamination and confirmed as negative

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

D- Animal Models

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

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Yes. This is described in Matierals and Methods section
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.	Yes. This is described in Matierals and Methods section
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Yes. This is described in Matierals and Methods section
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	Yes, we deposit our ChIP-seq data to GEO as GSE97972.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Yes to the extent of our knowledge
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. 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.	NA