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Aerobic glycolysis tunes YAP/TAZ transcriptional activity

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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.)

Editor: Thomas Schwarz-Romond

1st Editorial Decision 17 November 2014

Thank you very much for submitting your study that reveals an intriguing link between cellular metabolism and Yap/TAZ signaling for publication in The EMBO Journal.

I do enclose comments from three scientists that recognize value and confirm general interest in these findings. While overall encouraging, all three assessments converge on further validation of PFK1 as mediator of this glucose-dependent regulation, as well as some extensions on PFK1/Tead versus YAP/Tead interactions. Also, clarifications with regard to the employed YAP/TAZ signature should be made and the minor issues you find listed in the rather straightforward and explicit comments should be addressed.

Except for the first point that would need additional experimentation, I am certain that you would be in a strong position to respond in a relatively timely manner and I therefore kindly invite you to submit your final, revised study to your earliest convenience.

Please do not hesitate to get in touch, in case I can be of further assistance/to discuss feasibility, amount and timeline of necessary revisions.

I formally do have to remind you that The EMBO Journal only allows one single round of revisions.

Thank you very much for considering The EMBO Journal for presentation of your results. I look forward receiving your revised study and remain with best regards.

Referee #1:

In this MS, Enzo et al. show compelling data demonstrating a new interesting connection between cancer cell metabolism and YAP/TAZ activation. In particular they demonstrate that high rate of glycolysis can sustain YAP/TAZ pro-oncogenic functions and that, conversely, blunting glucose metabolism is a feasible strategy to block them. Mechanistically they found that Phosphofructokinase 1 (PFK1), the enzyme regulating the first committed step of glycolysis, binds TEADs transcription factors favouring their physical interaction with YAP/TAZ thus increasing their transcriptional activity. Importantly, in a large dataset of human breast cancers, glucose-driven gene expression is associated with YAP/TAZ activation and pathological stages.

This paper is straightforward and conclusions mostly follow the data, with very few exceptions. The results are of strong interest and provide significant new insights in understanding the molecular biology of the metabolic reprogramming of cancer cells.

The experiments are in general well planned and the figures are of good quality. Some points should be addressed in order to improve the strength of the data.

The following points need to be addressed:

1) Despite the authors claim that the glucose metabolism regulates YAP/TAZ transcriptional activity, the data shown in Fig. 1 are only correlative. Indeed, in any of the figures is demonstrated that the effect of 2DG or glucose on luciferase or gene expression, is mediated by YAP and/or TAZ. The most informative experiment would be a luciferase assay showing an increase of luciferase activity after glucose addiction to the medium, which should be prevented by silencing of YAP and TAZ.

2) It is not clear why, among the genes regulated by glucose through YAP/TAZ, the authors didn't identify the canonical CTGF, Cyr61 and Ankrd1, which are hallmarks of YAP/TAZ transcriptional activation. Please comment on this. Moreover, please provide information about Gene Ontology analysis of the genes in overlap between 2DG treatment and YAP/TAZ silencing.

3) In Fig. 2E please check the re-activation of oxydative phosphorylation after FH re-expression.

4) In Fig. 3A please add the silencing of AMPK as alternative to Compound C.

5) In Fig. 4 the authors should show the effect of 2DG on YAP, TAZ and TEADs protein and mRNA levels. Similarly, the authors should show YAP, TAZ and TEADs subcellular localization after 2DG treatment.

6) Show inputs in all the immunoprecipitation experiments.

7) In Figure 4A show also the effect of PFKFB3 overexpression upon YAP/TAZ silencing.

8) In Figure 5A please provide results relative also to secondary mammospheres. Moreover, the authors should show results from treatments with 2DG, siP, siG in Empty control.

Referee #2:

This manuscript investigates mechanisms through which glucose metabolism regulates gene expression in breast cancer cells. Microarray profiling of 2DG-treated cells showed enrichment for regulation of YAP/TAZ target genes. Interestingly, the mechanism through which glucose regulates YAP/TAZ involves PFK1 interaction with TEADs, thus promoting TEAD interaction and cooperation with YAP/TAZ. Glucose metabolism signature is associated with YAP/TAZ activation

in human breast tumors. Overall, this is a nicely done study that convincing demonstrates YAP/TAZ as a key effector of glucose-dependent gene regulation. With a few more experiments to clarify aspects the link between PFK1 and YAP/TAZ activation, the study should be suitable for publication in EMBO.

Major points
1. To clarify whether it is PFK1 interactions with TEADs, PFK1 glycolytic activity, or glycolytic-activity dependent interaction with TEADs that promote YAP/TAZ activity, a few questions should be addressed:
a. Can PFK1 knockdown-dependent suppression of YAP/TAZ activity and TEAD/YAP interaction be rescued with a downstream glycolytic metabolite such as pyruvate?
b. Does PFKFB3 expression promote PFK1 interaction with TEADs?
c. Does the PFK1 F2,6P mutant have impaired ability to activate YAP/TAZ?
2. Authors show that PFK1 is present in the nucleus. Where does the TEAD/PFK1 interaction occur, nucleus or cytoplasm? Does PFK1 localization change with glucose availability or with 2DG?

Minor points
1. For most experiments, authors do not show statistical analysis/ significance of their data. This analysis should be done to ensure that results are drawn from statistically significant results.
2. Why is TEAD4 used in some experiments and TEAD1 used in others?

Referee #3:

In this study, the authors investigate the regulation of the YAP/TAZ transcriptional regulators by glucose metabolism. They determine changes in gene expression following 2-deoxyglucose treatment in MCF10A breast epithelial and MDA-MB231 breast cancer cells. They found that glucose starvation leads to a reduction in genes from a YAP/TAZ signature. They perform experiments to exclude that this regulation is due to altered protein glycosylation, AMPK activity, LATS1/2 signalling upstream of YAP/TAZ or the mevalonate pathway. They next explored binding of proteins to a constitutive active form of YAP (5SA mutant) and identified PFK1 as a potential interaction partner. This binding was indirect and mediated by the TEAD binding partner of YAP/TAZ. They also show that glucose is essential for the interaction of YAP and TEAD and propose that binding of fructose-2,6-bisphosphate, an allosteric activator of PFK1, is essential for the function of PFK1 in modulating YAP-dependent gene expression. They also investigate the effect of PFK1 depletion on mammosphere formation induced by constitutively active TAZ in non-malignant breast epithelial cells, colony formation in breast cancer cells and induction of proliferation following the release from contact-inhibition. They also employ drosophila to demonstrate the effect of PFK1 silencing of Yki-dependent DIAP1 and Myc expression. Moreover, depletion of YAP/TAZ prevented glucose-dependent stimulation of proliferation in breast cancer cells and inhibition of YAP/TAZ binding to TEAD reduced colony formation in glycolytic cancer cells. Finally, the authors investigate evidence for a connection between glucose-dependent gene expression and YAP/TAZ in breast cancer.

Major comments:
Overall, the study includes a large amount of data and the line of thought behind the experiments is quite difficult to follow. Not all of this data supports the conclusions drawn by the authors. One issue is the definition of the YAP/TAZ signature used for the initial analysis (see specific comments). Moreover, would independently derived YAP signatures (e.g. those used in Calvo et al. 2013) score in this experiment? How do the enrichment scores compare to enrichment scores obtained against the complete set of gene sets within the MSigDB? Also, the genes shown in Fig. 1F are not classical YAP/TAZ target genes but show some overlap with target genes of E2F. The authors should investigate expression of ANKD1, CTGF and CYR61 in addition to the genes shown. Moreover, the mechanistic link between glucose metabolism, PFK1 and YAP/TAZ is not conclusive. The authors need to show the effect of glucose starvation or 2DG treatment on the interaction between PFK1 and TEAD, not just the effect of these treatments on YAP/TEAD interaction. Based on the experiments shown, it is not proven that PFK1 mediates the glucose-dependent interaction between YAP and TEAD. The authors should at least show that silencing of

PFK1 (or expression of the F2,6BP mutant) reduces the glucose dependent regulation of YAP/TEAD interaction. The data shown in the manuscript show glucose-dependent interaction between YAP and TEAD and interaction between PFK1 and TEAD but the two are not fully connected so far.
The authors state that PFK1 activity is proportional to the amount of glucose available through the activity of PFK2 enzymes. However, PFK2 enzymes are regulated by phosphorylation in response to signalling (e.g. via AMPK or Akt). It is therefore not clear how glucose starvation of 2DG treatment directly modulates F2,6BP availability or PFK1 activity. As this is the basis of the conclusions drawn from this study, this important connection needs to be experimentally confirmed. At a minimum, the authors need to show that the F2,6BP mutant of PFK1 blocks the effect of glucose and YAP/TEAD interaction. Moreover, nuclear localisation of PFK1 needs to be confirmed. It should also be considered whether PFK1 could alter phosphorylation of a component of the YAP/TEAD complex.
The data providing evidence of a link between glucose-metabolism and YAP/TAZ activity in breast cancer are not fully convincing. While the authors clearly go to some length to establish a comprehensive collection of breast cancer expression data, the subsequent statistical analysis is not entirely clear. The correlation coefficient between the two signatures is not high enough to demonstrate than a tentative link. Also, he authors should show individual signature scores for YAP signatures from different references rather than a combined score (see also comment below). This would allow a much cleaner evaluation of the strengths of the association between glucose-dependent gene expression and YAP/TAZ activity in breast cancer. Also, the number of genes left in the "Glucose NOT Yap" signature is rather small. Is this gene set still able to differentiate other glucose-dependent events that may not be linked to YAP (i.e. hypoxia signature)?

Specific comments:
The exact genes within the YAP/TAZ signature used for the analysis should be stated. The authors reference two earlier papers, which again reference a study (referenced as having been defined by YAP or TAZ overexpression in MCF10A cells, Zhang et al.). However, it is not clear which exact gene list has been used. This needs to be clarified.
Figure 1B should be represented as a box plot to represent the variation between different genes.
Compound C is highly unspecific (also blocks BMP signalling) and should not be used to test involvement of AMPK in YAP/TAZ regulation given the cross-talk between YAP/TAZ and Smad.
Basic reporter activity should have been enhanced following LATS silencing in Figure 3B.
Does the mutation of F2,6P binding in PFK1 alter the structure of the protein? Does it still show basal activity? Moreover, the expression of this mutant seems to be lower in Figure 4B compared to WT and could explain the lower pull down of TEAD4.
In Figure 4G and H, other mechanism of YAP regulation should be included as controls. Mutant PFK1 should also be used here.
Is the viability of MCF10A-MII cells affected by silencing of PFK1? Could this explain the reduction in mammosphere formation?
How specific is the TAP/TAZ inhibitor used in Figure 5I? How is YAP/TAZ TEAD interaction altered in these cells following different treatments?
It would be useful to add indicators of statistical significance into the figure where possible, particularly in Figure 6.
Scale bars are missing from the micrographs.
The text is very difficult to read and the figures need to be labelled more clearly.
Page numbers need to be added.

Additional correspondence (author) 20 November 2014

Thank you and the referees for the very rapid reviewing process. We are pleased to see that the referees appreciated the significance of our findings and the quality of data, and we are happy to be given the opportunity to revise the story. We will do all possible efforts to satisfy in full the points raised by the referees, and we believe we should be able to deliver the revised manuscript within the expected time limits.

As you pointed out, many experiments are straightforward. Please find hereafter a brief and general summary of how we plan to address the referees' comments:

i) we will address all the minor / technical points, and the requested additional controls.

ii) we will deepen our analysis of classical/direct YAP/TAZ target genes. It is true that in our array analysis CTGF did not appear coregulated, at least based on the microarray probes. This might be due to other inputs/transcription factors regulating CTGF gene expression independently from YAP/TAZ. But when we monitored CTGF-luciferase (i.e. using only the YAP/TAZ-responsive portion of the CTGF promoter) this was inhibited. To sort out the specific contribution of YAP/TAZ for the endogenous gene, we will try not only real-times, as requested, but also Chromatin IP for YAP/TAZ on the promoter of CTGF (we have the technique ready), and we expect YAP/TAZ ChIP-binding to be reduced upon 2DG treatment. In parallel, we will also try to perform a similar analysis on some putative TEAD-binding elements that we identified in the regulatory regions of some of the new genes that we presented. We think this will give a more complete analysis of the effects of glucose on YAP/TAZ transcriptional activity, and bring together functional with biochemical data.

iii) we will do the requested experiments to enrich our biochemistry/mechanistic insights. Basically the main questions are whether PFK1 localization or interaction with TEADs change depending on glucose (rev. 2 and 3), and to strengthen the proposed axis between PFKFB3, fructose-2,6-bisphosphate, and PFK1 in the regulation of TEAD/YAP interaction. We have all the reagents and experimental set-ups to try these experiments. Of course in this case it is not possible to predict the outcome of new experiments; bear in mind that we are not playing here with "classical" signaling regulators, such as protein kinases, ubiquitin ligases etc., but with key metabolic enzymes that lay at the center of very complex regulatory networks (indeed during the preparation of the manuscript we had to face the fact that some of the most established dogmas of the metabolism field, when challenged experimentally turn out to be based on very specific experiments, and limited to very particular cell types).

iv) we will clarify the points requested by rev. 3 on the bioinformatics analysis. This can be easily addressed, even if sometimes the referee was not very clear in his/her requests. Sometimes the impression is that this referee did not truly appreciate this kind of analyses, for example when he/she states that "the correlation coefficient is not high enough to demonstrate then a tentative link": in this kind of analysis one cannot judge "high enough", but just whether a correlation coefficient exists based on statistical analyses, indicating a potential positive or negative correlation between the two parameters (i.e. whether or not the hypothesis of a correlation is verified). In one point he/she mixed up the use of independent signatures with the fact that we used signature scores that sum up the expression values of multiple genes contained in one signature (when he/she states "the authors should show individual signature scores... rather than a combined score"). We are confident we will be able to manage these small issues and provide conclusive data to satisfy these points.

Thank you again for your help and advice.

Additional correspondence (editor) 20 November 2014

All your points are well taken, particularly the mechanistic ones to modulate key metabolic enzymes while assessing their biochemical/complex formation features.

I am pleased that you still consider attempting these and like to point out that we can be flexible in case the results reveal further-reaching complexity/turn out to be not as straightforward.

I certainly keep the fingers crossed for efficient pursuit of the relevant/indicated experimentation. Please keep me posted in a timely manner to not delay further consideration beyond the three month revision period.

Thanks once more for engaging/considering EMBO for presentation of your data.

1st Revision - authors' response 07 February 2015

Response to Reviewers – manuscript EMBOJ-2014-90379

Referee #1:

In this MS, Enzo et al. show compelling data demonstrating a new interesting connection between cancer cell metabolism and YAP/TAZ activation. In particular they demonstrate that high rate of glycolysis can sustain YAP/TAZ pro-oncogenic functions and that, conversely, blunting glucose metabolism is a feasible strategy to block them. Mechanistically they found that Phosphofructokinase 1 (PFK1), the enzyme regulating the first committed step of glycolysis, binds TEADs transcription factors favoring their physical interaction with YAP/TAZ thus increasing their transcriptional activity. Importantly, in a large dataset of human breast cancers, glucose-driven gene expression is associated with YAP/TAZ activation and pathological stages. This paper is straightforward and conclusions mostly follow the data, with very few exceptions. The results are of strong interest and provide significant new insights in understanding the molecular biology of the metabolic reprogramming of cancer cells.
The experiments are in general well planned and the figures are of good quality. Some points should be addressed in order to improve the strength of the data.

We thank the Reviewer for the positive comments on the general interest and technical soundness of our data. In this revised manuscript we provide a series of new experiments that we have performed following her/his suggestions that we think strengthened our conclusions. We hope the Reviewer will now find the manuscript suitable for publication.

The following points need to be addressed:
1) Despite the authors claim that the glucose metabolism regulates YAP/TAZ transcriptional activity, the data shown in Fig. 1 are only correlative. Indeed, in any of the figures is demonstrated that the effect of 2DG or glucose on luciferase or gene expression, is mediated by YAP and/or TAZ. The most informative experiment would be a luciferase assay showing an increase of luciferase activity after glucose addiction to the medium, which should be prevented by silencing of YAP and TAZ.

Thanks for the suggestion. We performed an experiment where we show that YAP/TAZ activity that has been previously inhibited upon 2DG treatment can be reactivated by providing fresh medium (new Supplementary Figure 1E). So, untreated cells can be compared to cells where glucose metabolism has been shut down and then reactivated. Moreover, this reactivation is dependent on YAP/TAZ (new Supplementary Figure 1E). We also provide similar evidence by showing that increased luciferase activity induced by glucose in MDA-MB-231 cells previously fed on galactose depends on YAP/TAZ (new Supplementary Fig. 2G).

2) It is not clear why, among the genes regulated by glucose through YAP/TAZ, the authors didn't identify the canonical CTGF, Cyr61 and Ankrd1, which are hallmarks of YAP/TAZ transcriptional activation. Please comment on this.

Some established YAP/TAZ target genes (such as *CTGF* and *ANKRD1*) were not identified among the genes commonly regulated by YAP/TAZ and glucose metabolism in the microarrays. To double-check this we performed real-time PCR for *CTGF* and *ANKRD1*, and again failed to observe any convincing downregulation upon 2DG treatment (now shown in Supplementary Fig. 1S). However, we found at least another recently identified direct YAP/TAZ target gene, *HMMR/RHAMM*

(Wang et al., PNAS 2014), downregulated in the microarrays (Table 3) and by RTPCR (Fig. 1F), in line with our model.

Given these different responses, we sought to reinforce the notion that glucose metabolism directly regulates YAP/TAZ transcriptional activity (see also point #1). To this end we performed chromatin-immunoprecipitation experiments, enabling us to visualize the specific engagement of selected promoters by YAP/TAZ, in control cells vs. in cells with inhibited glucose metabolism. We monitored both genes coregulated by glucose and YAP/TAZ, such as *HMMR* and *TK1* (coming from our microarray analysis, and for which a TEAD-binding element was already known or easily recognizable – see methods), and genes apparently not regulated by glucose, such as *CTGF* and *ANKRD1*. As shown in Fig. 4C and Supplementary 4G, the binding of YAP to the promoter of these genes, including *CTGF* and *ANKRD1*, was downregulated upon treatment of cells with 2DG, both in MDA-MB-231 and in MCF10A cells. This strongly indicates that the specific contribution of YAP/TAZ at these promoters is sustained by glucose.

To further validate these findings, we then isolated the promoter segments of *CTGF* and *TK1* spanning the predicted TEAD-binding sites, in order to restrict our analysis to YAP/TAZ-induced transcription; in line with ChIP data, these promoters coherently respond to YAP/TAZ silencing, to PFK1 silencing and to 2DG treatment by luciferase assays (see Supplementary Fig. 1G, Supplementary Fig. 3I and Supplementary Fig. 4H-I).

We conclude from these experiments that, at least for the genes here analyzed, glucose metabolism sustains the formation of YAP/TEAD transcriptional complexes, indicating a direct effect of glucose on YAP/TAZ transcriptional activity. This nicely complements previous data showing inhibition of YAP/TEAD1 protein-protein interaction. We speculate that the absence of regulation of *CTGF* and *ANKRD1* mRNA might be due to activation of parallel/alternative pathways upon inhibition of glucose metabolism (in this regard, please consider that every gene is potentially subjected to multiple combinatorial regulations).

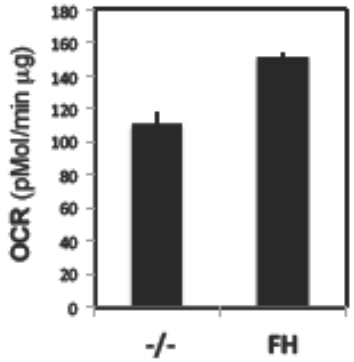
Moreover, please provide information about Gene Ontology analysis of the genes in overlap between 2DG treatment and YAP/TAZ silencing.

We provide this information in Supplementary Figure 1U. Gene Ontology analysis indicates a significant enrichment of genes involved in cell cycle regulation, stress response / DNA damage, and DNA metabolism / replication. Regulation of cell cycle genes is in accordance with the known effects of both glucose and YAP/TAZ on cell proliferation, and fits with our data on cell proliferation shown in Fig. 5. Regulation of nucleotide metabolism and DNA replication is also in line with these effects, and with the finding that YAP/TAZ preferentially regulate G1/S transition (see Fig. 5 and many other publications); moreover, this observation is at least in part validated by transcriptional analysis performed on *TK1* (*thymidine kinase*), *TYMS* and *RRM2*, key genes involved in DNA synthesis. These observations suggest an unexpected function of YAP/TAZ as core regulators of the DNA replication machinery.

3) In Fig. 2E please check the re-activation of oxydative phosphorylation after FH re-expression.

We checked for differential sensitivity of UOK cells to glycolysis inhibition in the first submission already; parental cells rely very strongly on glycolysis, as shown by higher sensitivity to 2DG treatment (shown in Fig. 5H). This is in line with the inability of these cells, unless reconstituted of FH, to grow in galactose (Sudarshan et al., Mol Cell Biol 2009).

We performed the requested measurements. We provide the results for the Referee only here below because these have been already published by Dr. Linehan who provided us with the cells (Yang et al., 2013; Sudarshan et al., 2009).



4) In Fig. 3A please add the silencing of AMPK as alternative to Compound C.

This experiment is now provided in the new Supplementary Fig. 3D, showing that AMPK silencing is not sufficient to rescue YAP/TAZ activity upon 2DG treatment. For this experiment, we used the very same siRNA oligos published by DeRan et al., Cell Reports 2014, and re-validated in our hands by western-blotting (also shown in Supplementary Fig. 3E).

5) In Fig. 4 the authors should show the effect of 2DG on YAP, TAZ and TEADs protein and mRNA levels. Similarly, the authors should show YAP, TAZ and TEADs subcellular localization after 2DG treatment.

We performed the requested experiments. We observed no major variations in the mRNA (not shown) or protein expression levels of YAP, TAZ, TEADs, LATS and PFK1 (new Supplementary Fig. 3F). We detected a slight increase in the phosphorylation of YAP S127, perhaps in keeping with DeRan et al., but this is minor if compared with other treatments inducing S127 phosphorylation, such as Rho or Factin inhibition (not shown).
We also checked by immunofluorescence the localization of YAP/TAZ and TEAD1 (new Supplementary Fig. 3G and Supplementary Fig. 4A): while TEAD1 remains fully nuclear upon 2DG treatment, we could detect a slight cytoplasmic relocalization of YAP/TAZ (we used Cerivastatin treatment as positive control for a more quantitative nuclear exclusion – see Sorrentino et al., NCB 2014).

6) Show inputs in all the immunoprecipitation experiments.

Where possible, this has been amended. In all other cases, please consider that we stated in the methods section that immunoprecipitations were always performed after normalization by Bradford assay for equal protein content, and that we did not observe significant changes in YAP, TEADs or PFK1 protein levels in our lysates upon 2DG treatments (see response to point #5), ruling out potential problems arising from unequal loadings in the co-IPs.

7) In Figure 4A show also the effect of PFKFB3 overexpression upon YAP/TAZ silencing.

We attempted the experiment and found to our surprise that YAP/TAZ silencing is very toxic in 293 cells, leading to almost complete cell death of siRNA-transfected cells in the time frame required for the experiment requested. To provide alternative evidence that the PFKFB3 overexpression is specific for YAP/TAZ, we developed a

luciferase reporter bearing the same minimal promoter of the 8XGTIIC-lux, but lacking of the multimerized TEAD-binding sites (delta8XGT-lux). This reporter lacks of the YAP/TAZ responsive region, and indeed in MDA-MB-231 cells the delta8X reporter is not responsive to YAP/TAZ silencing or NF2/merlin overexpression (Supplementary Fig. 3P). We then repeated overexpression of PFKFB3 in 293 cells, and we now show in Fig. 3K that gain of PFKFB3 increases specifically the activity of the 8XGTIIC-lux reporter, leaving unmodified the delta8XGT-lux.

8) In Figure 5A please provide results relative also to secondary mammospheres. Moreover, the authors should show results from treatments with 2DG, siP, siG in Empty control.

This is provided in the new Supplementary Fig. 5B and 5C.
Finally, please note that in response to Reviewer #3 we enriched our bioinformatics analyses by including independent YAP/TAZ signatures both in the initial GSEA (Fig. 1A and Supplementary Table 1) and in the analysis on mammary tumors provided in Figure 6, all showing coherent results.

Referee #2:

This manuscript investigates mechanisms through which glucose metabolism regulates gene expression in breast cancer cells. Microarray profiling of 2DGtreated cells showed enrichment for regulation of YAP/TAZ target genes. Interestingly, the mechanism through which glucose regulates YAP/TAZ involves PFK1 interaction with TEADs, thus promoting TEAD interaction and cooperation with YAP/TAZ. Glucose metabolism signature is associated with YAP/TAZ activation in human breast tumors. Overall, this is a nicely done study that convincing demonstrates YAP/TAZ as a key effector of glucosedependent gene regulation. With a few more experiments to clarify aspects the link between PFK1 and YAP/TAZ activation, the study should be suitable for publication in EMBO.

We thank the Reviewer for his/her positive comments on our study. In this revised manuscript, we provide new experiments that went at the heart of the Reviewer's comments, and that we believe strengthened our conclusions. We hope the Reviewer will now find the manuscript suitable for publication.

Major points

1. To clarify whether it is PFK1 interactions with TEADs, PFK1 glycolytic activity, or glycolytic-activity dependent interaction with TEADs that promote YAP/TAZ activity, a few questions should be addressed:
a. Can PFK1 knockdown-dependent suppression of YAP/TAZ activity and TEAD/YAP interaction be rescued with a downstream glycolytic metabolite such as pyruvate?
b. Does PFKFB3 expression promote PFK1 interaction with TEADs?
c. Does the PFK1 F2,6P mutant have impaired ability to activate YAP/TAZ?

We performed the pyruvate experiment upon PFK1 silencing, but we did not observe any increase of YAP/TAZ activity upon adding pyruvate; similar results (i.e. no rescue) were obtained by supplementing pyruvate to 2-deoxyglucose or by growing cells in galactose + pyruvate. This is now provided in Supplementary Fig. 2H, and discussed as data not shown in page 9: …*Remarkably, knockdown of PFK1 with two independent siRNAs caused inhibition of YAP/TAZ activity in MDA-MB-231 cells (Fig. 3E and Supplementary Fig. 3I). Also in this case, LATS1/2 siRNA, mevalonate or pyruvate could not rescue inhibition of YAP/TAZ activity upon PFK1 knockdown*… These results suggest that providing the last metabolic intermediate of glycolysis is not sufficient to rescue YAP/TAZ activity.

We tested the second experiment but unfortunately failed to observe any enhancement in the endogenous PFK1/TEAD1 interaction, perhaps due to inefficient or uneven transfection of PFKFB3 plasmid. However, in response to Reviewer #1 we performed better controls on the specificity of PFKFB3 effects, and now show that PFKFB3 enhances YAP/TAZ transcriptional activity in a manner that is specific for the TEADbinding sites of the luciferase reporter used (new Figure 3K and Supplementary Fig. 3P).

We also tried to overexpress PFK1 isoforms both in 293 and MDA-MB-231 cells but we failed to observe either upregulation of downregulation of YAP/TAZ activity. This might be due to non-limiting amounts of PFK1 in the cells, or to endogenous feedback mechanisms precisely limiting the active subpool of PFK1.

To anyway reinforce the notion that PFK1 is required for YAP/TEAD1 interaction, we now provide a co-immunoprecipitation experiment showing that glucose-induced YAP/TEAD1 binding is dependent on endogenous PFK1 levels (new Fig. 4D). Collectively, these results are compatible with the simple idea that PFK1 performs some sort of scaffolding activity for TEADs and YAP/TAZ.

Please finally note that, in response to Reviewer #1 and #3, we performed a new set of experiments providing better evidence that glucose metabolism directly regulates YAP/TAZ transcriptional activity:
i) we now include new endogenous targets jointly regulated by glucose and YAP/TAZ in Figure 1F and Supplementary Figure 1S, including the established YAP/TAZ target *RHAMM/HMMR*;
ii) we show by chromatin immunoprecipitation experiments that glucose regulates the recruitment of YAP to a series of established and new target gene promoters (*CTGF*, *ANKRD1*, *RHAMM/HMMR* and *TK1*) (Figure 4C and Supplementary Fig 4G);
iii) we also provide new luciferase assays showing that the promoter elements of the *CTGF* and *TK1* genes, encompassing the TEAD-binding sites used in the ChIP analysis, respond coherently to YAP/TAZ and glucose metabolism (see Supplementary Fig. 1G, Supplementary Fig. 3I and Supplementary Fig. 4H-I).

Taken together, we think these are important piece of information to causally link glucose metabolism and YAP/TAZ.

2. Authors show that PFK1 is present in the nucleus. Where does the TEAD/PFK1 interaction occur, nucleus or cytoplasm? Does PFK1 localization change with glucose availability or with 2DG?

We now show by PLA (proximity ligation assay) that it is possible to detect a specific interaction of endogenous PFK1 and TEAD1 proteins in the nuclear compartment. The description of this experiment is now incorporated in the new Supplementary Figure 3O.

We tested the effect of 2DG on PFK1 localization but we did not observe significant differences (not shown). In response to Reviewer #1 we checked also YAP and TEAD1 subcellular localization (Supplementary Figures 3G and 4A), and in the case of YAP we observed a mild cytoplasmic exclusion, although not comparable to that caused by other treatments such as Cerivastatin (and inhibitor of mevalonate metabolism – Sorrentino et al., NCB 2014).

Minor points
1. For most experiments, authors do not show statistical analysis/ significance of their data. This analysis should be done to ensure that results are drawn from statistically significant results.

This is now provided.

2. Why is TEAD4 used in some experiments and TEAD1 used in others?

The trivial reason is that our TEAD1 expression plasmid is FLAG-tagged and our TEAD4 expression plasmid is MYC-tagged; so when we co-immunoprecipitate PFK1, which is FLAG-tagged, we cannot use a FLAG-tagged TEAD.
From a more general point of view, using TEAD1 or TEAD4 should make no significant difference. The crystal structure between YAP and TEADs was originally solved by using either TEAD4 or TEAD1, with overlapping results (Chen et al., Genes&Dev 2010; Li et al., Genes&Dev 2010). Genetically, TEAD4 shows a very early requirement in the blastocyst (Nishioka et al., Dev. Cell 2009), while TEAD1 is required later during embryogenesis, acting in a redundant manner with TEAD2 (Sawada et al., Mol. Cell Biol. 2008). This might indicate that TEADs have redundant functions, and their requirements were observed in different tissues likely because of different expression levels. To show this in our cells, we performed an experiment with TEADs siRNAs, showing that TEAD1 and TEAD4, together with lower expression levels of TEAD2 and TEAD3, are required for YAP/TAZ driven luciferase expression (Supplementary Fig. 3L and 3M).

Finally, please note that in response to Reviewer #3 we enriched our bioinformatics analyses by including independent YAP/TAZ signatures both in the initial GSEA and in the analysis on mammary tumors provided in Figure 6. We also included as Supplementary material a detailed list of the genes contained in the YAP/TAZ signatures for completeness and clarity.

Referee #3:

In this study, the authors investigate the regulation of the YAP/TAZ transcriptional regulators by glucose metabolism. They determine changes in gene expression following 2-deoxyglucose treatment in MCF10A breast epithelial and MDA-MB231 breast cancer cells. They found that glucose starvation leads to a reduction in genes from a YAP/TAZ signature. They perform experiments to exclude that this regulation is due to altered protein glycosylation, AMPK activity, LATS1/2 signalling upstream of YAP/TAZ or the mevalonate pathway. They next explored binding of proteins to a constitutive active form of YAP (5SA mutant) and identified PFK1 as a potential interaction partner. This binding was indirect and mediated by the TEAD binding partner of YAP/TAZ. They also show that glucose is essential for the interaction of YAP and TEAD and propose that binding of fructose-2,6-bisphosphate, an allosteric activator of PFK1, is essential for the function of PFK1 in modulating YAP-dependent gene expression. They also investigate the effect of PFK1 depletion on mammosphere formation induced by constitutively active TAZ in non-malignant breast epithelial cells, colony formation in breast cancer cells and induction of proliferation following the release from contact-inhibition. They also employ drosophila to demonstrate the effect of PFK1 silencing of Yki-dependent DIAP1 and Myc expression. Moreover, depletion of YAP/TAZ prevented glucose-dependent stimulation of proliferation in breast cancer cells and inhibition of YAP/TAZ binding to TEAD reduced colony formation in glycolytic cancer cells. Finally, the authors investigate evidence for a connection between glucose-dependent gene expression and YAP/TAZ in breast cancer.

Major comments:
Overall, the study includes a large amount of data and the line of thought behind the experiments is quite difficult to follow. Not all of this data supports the conclusions drawn by the authors. One issue is the definition of the YAP/TAZ signature used for the initial analysis (see specific comments). Moreover, would independently derived YAP signatures (e.g. those used in Calvo et al. 2013) score in this experiment?

This issue is also recalled in the specific comment here below:

Specific comments:
The exact genes within the YAP/TAZ signature used for the analysis should be stated. The authors reference two earlier papers, which again reference a study (referenced as having been defined by YAP or TAZ overexpression in MCF10A cells, Zhang et al.). However, it is not clear which exact gene list has been used. This needs to be clarified.

The signature used for the initial analysis derives from Zhang et al. JBC 2009 as correctly pointed out from the reviewer, and has been used in two previous publications from our group (Dupont et al., Nature 2011; Cordenonsi et al., Cell 2011) and also in Calvo et al., NCB 2013 (where it was called "induced by YAP and TAZ").

We now cite the original publication in the methods, and provide the exact gene list as Supplementary Table 7.

As requested, we repeated the GSEA analysis by including other independently derived YAP signatures (new Fig. 1A). We now provide:

- the YAP/TAZ signature from Zhang et al. JBC 2009, used in the old Fig. 1A (this signature is the "induced by YAP and TAZ" found in Calvo et al.);
- a signature containing the genes induced by YAP overexpression from Zhao et al. Genes&Dev 2008 ("induced by YAP" signature found in Calvo et al.);
- the YAP signature used in Dupont et al., Nature 2011 ("YAP/TAZ" signature in Calvo et al.);

All these signatures display a significant enrichment among the genes inhibited by 2DG treatment, in line with previous results. This is also evident by looking at the average expression of the signatures in control and 2DG-treated samples (new Supplementary Fig. 1A).

Moreover, we also included in the analysis a signature containing the genes INHIBITED by YAP overexpression (from Zhao et al., 2008), and this signature displays a significant enrichment among the genes ACTIVATED by 2DG treatment (i.e. inhibited by glucose metabolism). Thus, we can find a correlation between YAP/TAZ activity and glucose metabolism by looking both at activated and inhibited genes.

We provide the exact gene lists for all the signatures in Supplementary Table 7.

How do the enrichment scores compare to enrichment scores obtained against the complete set of gene sets within the MSigDB?

We understand here that the Reviewer is asking how the YAP/TAZ signature and the other signatures that we used for the bioinformatics analysis in Fig. 1A would perform compared to other gene sets present in the MSigDB. For this, we repeated our GSEA analysis by including all the BIOCARTA gene sets, encompassing more than 200 gene sets (http://www.broadinstitute.org/gsea/msigdb/collections.jsp); these gene sets are organized in "pathways", each containing a collection of genes involved in a particular biological process (for example, they are involved in "cell cyle", "fibrinolysis" or "mitochondria"). The results are provided in Supplementary Fig. 1U. We found a significant enrichment of the following BIOCARTA gene sets among the genes inhibited by 2-deoxyglucose treatment:

MCM pathway (i.e. genes involved in the pre-replication complex and in the initiation of the DNA replication origins)
PROTEASOME
P27 (i.e. genes involved in the regulation of the CDK inhibitor P27 and thus of the G1/S transition)
RANMS (i.e. genes involved in the regulation of Ran and mitotic spindle)

ATRBRCA (i.e. genes related to the role of BRCA1, BRCA2 and ATR in DNA repair and cancer susceptibility)
CELL_CYCLE
RB (Retinoblastoma)
CDC42RAC

The enrichment of gene sets related to cell-cycle regulation, G1/S transition and DNA synthesis/repair likely reflects the effects of glucose metabolism on proliferation in our cellular systems.

Of note, in response to Reviewer #1 (see her/his point #2) we performed an analysis of the genes commonly regulated by YAP/TAZ and by glucose metabolism based on GO (Gene Ontology) categories, and found also in this case a significant enrichment in cell-cycle and DNA metabolism genes. These results fit well with the validation of *TK1*, *TYMS* and *RRM2* (key enzymes involved in DNA synthesis) shown in Fig. 1F and Supplementary Fig. 1S.

Also, the genes shown in Fig. 1F are not classical YAP/TAZ target genes but show some overlap with target genes of E2F.

Among the genes that we validated, some are also known to be E2F targets. Indeed we found a significant enrichment of the E2F signature and of the Biocarta Retinoblastoma pathway (see the previous point) in our GSEA analysis. We performed some experiments on E2F in the initial submission, and we showed that both 2DG and YAP/TAZ regulate E2F-driven luciferase transcription (Supplementary Fig. 1L); this might account for the presence of the E2F signature in the GSEA.

In the same set of data we also showed that inducing growth arrest (by overexpressing classical CDK inhibitors such as p21 and p27) could inhibit E2F, but not YAP/TAZ activity (compare Supplementary Fig. 1L and 1M). We included these data on purpose to exclude that the regulation of YAP/TAZ upon 2DG treatment was indirectly due to growth arrest.

An alternative explanation for this overlap is the observation, recently proposed by several authors, that Hippo and Retinoblastoma/E2F pathway are intimately linked (Ehmer Cell Reports 2014; Nicolay Genes&Dev 2011; Kapoor et al., Cell 2014). Noteworthy, in some cases it was even proposed that YAP and E2F factors cooperate at the promoter level to activate genes essential for cell-cycle progression; thus, it is often hard to untangle whether a gene is being regulated by one or the other factor (i.e. some E2F target genes are also direct YAP/TAZ target genes). The new analysis on *TK1* transcription (discussed in details as response to the following point) is an example of an established E2F target gene that is also a direct YAP/TAZ target.

The authors should investigate expression of ANKD1, CTGF and CYR61 in addition to the genes shown.

Some established YAP/TAZ target genes (such as *CTGF* and *ANKRD1*) were not identified among the genes commonly regulated by YAP/TAZ and glucose metabolism in the microarrays. To double-check this we performed real-time PCR for *CTGF* and *ANKRD1*, and again failed to observe any convincing downregulation upon 2DG treatment (now shown in Supplementary Fig. 1S). However, we found at least another recently identified direct YAP/TAZ target gene, *HMMR/RHAMM* (Wang et al., PNAS 2014), downregulated in the microarrays (Table 3) and by RT11 PCR (Fig. 1F), in line with our model.

Given these different results, we sought to reinforce the notion that glucose metabolism directly regulates YAP/TAZ transcriptional activity. To this end we performed chromatin-immunoprecipitation experiments, enabling us to visualize the specific engagement of selected promoters by YAP/TAZ, in control cells vs. in cells with inhibited glucose metabolism. We monitored both genes co-regulated by glucose

and YAP/TAZ, such as *HMMR* and *TK1* (coming from our microarray analysis, and for which a TEAD-binding element was already known or easily recognizable – see methods, "reagents" section, where we provide details about TK1 promoter, and Supplementary Table 6 where we provide the PCR oligos for ChIP), and genes apparently not regulated by glucose, such as *CTGF* and *ANKRD1*. As shown in Fig. 4C and Supplementary 4G, the binding of YAP to the promoter of these genes, including *CTGF* and *ANKRD1*, was downregulated upon treatment of cells with 2DG, both in MDA-MB-231 and in MCF10A cells. This strongly indicates that the specific contribution of YAP/TAZ at these promoters is sustained by glucose. Please note that *TK1* is also one of the classical E2F target genes, but ChIP analys of YAP/TAZ rules out an indirect regulation through E2F.

To further validate these findings, we then isolated the promoter segments of *CTGF* and *TK1* spanning the predicted TEAD-binding sites, in order to restrict our analysis to YAP/TAZ-induced transcription; in line with ChIP data, these promoters coherently respond to YAP/TAZ silencing, to PFK1 silencing and to 2DG treatment by luciferase assays (see Supplementary Fig. 1G, Supplementary Fig. 3I and Supplementary Fig. 4H-I).

We conclude from these experiments that, at least for the genes here analyzed, glucose metabolism sustains the formation of YAP/TEAD transcriptional complexes, indicating a direct effect of glucose on YAP/TAZ transcriptional activity. This nicely complements previous data showing inhibition of YAP/TEAD1 protein-protein interaction. We speculate that the absence of regulation of *CTGF* and *ANKRD1* mRNA might be due to activation of parallel/alternative pathways upon inhibition of glucose metabolism, acting on distinct enhancer regions. In this regard, please consider that every gene is potentially subjected to multiple combinatorial regulations; for example, *CTGF* and *ANKRD1* were recently described as direct targets of MAL/SRF (Esnault et al., Genes&Development 2014).

Moreover, the mechanistic link between glucose metabolism, PFK1 and YAP/TAZ is not conclusive. The authors need to show the effect of glucose starvation or 2DG treatment on the interaction between PFK1 and TEAD, not just the effect of these treatments on YAP/TEAD interaction. Based on the experiments shown, it is not proven that PFK1 mediates the glucosedependent interaction between YAP and TEAD. The authors should at least show that silencing of PFK1 (or expression of the F2,6BP mutant) reduces the glucose dependent regulation of YAP/TEAD interaction. The data shown in the manuscript show glucose-dependent interaction between YAP and TEAD and interaction between PFK1 and TEAD but the two are not fully connected so far.

We now show, also in response to other Reviewers, that glucose-induced YAP/TEAD1 binding is dependent on endogenous PFK1 levels (Fig. 4D). We think this is an important piece of information that complements previous data obtained by using the PLA technique and now fully support our claim that PFK1 is required for glucose-induced YAP/TEAD interaction.

The authors state that PFK1 activity is proportional to the amount of glucose available through the activity of PFK2 enzymes. However, PFK2 enzymes are regulated by phosphorylation in response to signalling (e.g. via AMPK or Akt). It is therefore not clear how glucose starvation of 2DG treatment directly modulates F2,6BP availability or PFK1 activity. As this is the basis of the conclusions drawn from this study, this important connection needs to be experimentally confirmed. At a minimum, the authors need to show that the F2,6BP mutant of PFK1 blocks the effect of glucose and YAP/TEAD interaction. Moreover, nuclear localisation of PFK1 needs to be confirmed.

In our paragraph introducing the PFK1 mutant, we just wanted to make clear that PFK1 activity is regulated, among several different mechanisms, by F2,6BP produced

by PFKFB enzymes. Since F2,6BP directly derives from F6P, higher levels of glycolysis and thus an enhanced production of F6P may also facilitate F2,6BP production. In particular cellular systems and upon select treatments, the activity of PFKFB enzymes can be also modulated by other inputs, for example by AMPK and AKT as indicated by the Reviewer; however, our data excluded a direct involvement of these pathways. Part of these data has been now moved to Figure 4, and the discussion has been simplified to avoid overstatements or confusion (page 10, end of 1st paragraph).

We tried to test a dominant-negative effect of the PFK1 mutant in our assays, but failed; this is likely due inability of overexpressed PFK1 mutant to interfere with endogenous PFK1. As for the nuclear localization of PFK1/TEAD interaction, we now provide a PLA assay showing a specific nuclear signal (Supplementary Fig. 3O).

It should also be considered whether PFK1 could alter phosphorylation of a component of the YAP/TEAD complex.

This was a nice suggestion. Indeed other have shown that a metabolic enzymes such as Pyruvate Kinase 2 can also act as protein-kinase to regulate transcription, for example by modifying Histone3 or beta-catenin. We performed this experiment using immunopurified FLAG-PFK1 and tested whether it might promote radioactive 32PPhosphate incorporation onto GST-YAP or GST-TEAD, but we could not detect any incorporation above background (GST alone).

As response to Reviewer #1, we checked YAP phosphorylation and subcellular localization in cells upon 2DG treatment, but we could detect only a very minor phosphorylation and almost no nuclear exclusion, if compared with Cerivastatin treatment – a positive control causing YAP inhibition and quantitative nuclear exclusion in a phosphorylation-dependent manner (Sorrentino et al., NCB 2014) (Supplementary Figures 3F and G).

The data providing evidence of a link between glucose-metabolism and YAP/TAZ activity in breast cancer are not fully convincing. While the authors clearly go to some length to establish a comprehensive collection of breast cancer expression data, the subsequent statistical analysis is not entirely clear.
The correlation coefficient between the two signatures is not high enough to demonstrate than a tentative link. […] This would allow a much cleaner evaluation of the strengths of the association between glucose-dependent gene expression and YAP/TAZ activity in breast cancer.

We respectfully disagree. In this type of analysis, it is not possible to judge whether a correlation is "high enough". A value of R2=0,731 for the coefficient of determination, as in our case, is clearly different from R2=0 (expected in the absence of any linear correlation) and this difference is highly meaningful based on the pvalue ($p<0.0001$). Anyway, we now provide the most relevant parameter, i.e. Pearson ρ correlation (0,855) and the p-value for this ($p<0.0001$). The slope of the regression line, shown in red in Fig. 6A, is now indicated for completeness (0.532). So, our data support the idea of a positive correlation between genes regulated by YAP/TAZ and by glucose metabolism in an unprecedented database of primary human breast cancers.

Please note that we never intended to claim any causal relationship based on these bioinformatics analyses; this was evident in the statement closing this paragraph: *"the data indicates that during mammary tumor progression metabolic reprogramming toward aerobic glycolysis is accompanied by elevated YAP/TAZ activity."*

Also, the authors should show individual signature scores for YAP signatures from different references rather than a combined score (see also comment

below).

The signature score that we are using is not a combined score of several different signatures, but a unique score generated by the combination of the single scores for each gene contained in the YAP/TAZ signature. We explained in the methods that: *Signature scores have been obtained summarizing the standardized expression levels of signature genes into a combined score with zero mean (Adorno et al, 2009). The values shown in graphs are thus adimensional.*

We also repeated the correlation analysis by using another YAP/TAZ signature ("induced by YAP" from Zhao et al., 2008), and show a comparable positive correlation (now in Supplementary Fig. 6A and 6B).

Also, the number of genes left in the "Glucose NOT Yap" signature is rather small. Is this gene set still able to differentiate other glucose-dependent events that may not be linked to YAP (i.e. hypoxia signature)?

We explored whether the "Glucose NOT Yap" signature was able to differentiate other signaling events than YAP/TAZ, by performing an analysis similar to the one carried out in Fig. 6B. We classified the patients of the breast cancer dataset according to "high" or "low" expression of the "Glucose NOT Yap" signature, and then asked if any of the signatures used for the GSEA was differentially expressed (by looking at the overall expression levels of each signature), but we found no significant differences.

We could not attempt to differentiate other glucose-dependent events that may not be linked to YAP, because we are not aware of other published glucose-dependent transcriptional events relevant to breast cancer progression to extract a reliable gene signature. We don't think this case may apply to HIF/hypoxia because this is an inducer of glycolysis, among other phenotypes, and not a glucose-dependent event.

That said, we cannot assume that the "Glucose NOT Yap" signature is in principle meaningless just because it is "rather small" (27 genes). In this type of bioinformatics analyses, it is common to find similar analyses based on expression of even one single gene (see for example Onodera and Bissell, JCI 2014); moreover, in our experience a signature based on as little as two genes (the "minimal signature" of Adorno et al., Cell 2009) has a performance comparable to an established and clinically validated 70-gene signature such as the "mammaprint" (Glas et al., BMC Genomics 2006).

Figure 1B should be represented as a box plot to represent the variation between different genes.

The Reviewer asks for a representation of the variation between different genes, but these plots do not directly indicate the expression levels of genes, but the module average +- SEM. We explained this in the methods: *Average signature expression has been calculated as the standardized average expression of all signature genes in sample subgroups (e.g., 2DG treated/controls; histological grade).*

This type of representation is a standard in the field, see for example Kim et al., A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs. Cell. 2010 Oct 15;143(2):313-24.

We have now made this clearer in the figure legend to avoid confusion.

Compound C is highly unspecific (also blocks BMP signalling) and should not be used to test involvement of AMPK in YAP/TAZ regulation given the crosstalk between YAP/TAZ and Smad.

We now show that AMPKalpha1/2 silencing is not sufficient to rescue YAP/TAZ activity upon 2DG treatment (Supplementary Figure 3D). For this experiment, we

used the very same siRNA oligos published by DeRan et al., Cell Reports 2014 and re-validated in our hands by western-blotting (Supplementary Figure 3E).
We also checked whether inhibition of BMP signaling alone had any effect on YAP/TAZ activity by supplementing recombinant Noggin in the culture medium but nothing changed (not shown). As internal control, the same treatment was sufficient to inhibit exogenously added BMP2 induction of the established Smad1 reporter ID1-BRE-luciferase reporter.

Basic reporter activity should have been enhanced following LATS silencing in Figure 3B.

This is true only if LATS kinases were highly active in basic conditions in MDA-MB-231, but these cells are mutated in the NF2/merlin gene (upstream activator of LATS), and display low levels of YAP phosphorylation (a direct read-out of LATS activity). Indeed we show that once these cells are reconstituted of NF2/Merlin expression, YAP/TAZ activity is dramatically inhibited, and now LATS silencing dominates over NF2 and brings back reporter activity to basal levels (see also Aragona et al., 2013; Sorrentino et al., 2014). This is the best functional internal control we can provide. Please note that LATS silencing with the very same reagents does induce a clear enhancement of basal expression of endogenous YAP/TAZ target genes in MCF10A cells (see Dupont et al., 2011; Aragona et al., 2013), where basal phosphorylation levels of YAP (and thus, of LATS activity) are much higher. These control references are indicated in the text and methods.

Does the mutation of F2,6P binding in PFK1 alter the structure of the protein? Does it still show basal activity?

The mutations that we have introduced are very localized, and we took care to introduce Alanine mutations instead of more bulky/charged aminoacids, so we should not expect any major disruption of protein folding. Moreover, although the PFK1 mutant shows reduced binding to TEAD4 (see below the response to another point), it is still able to bind it, indicating that the protein is not completely crippled.

We attempted a direct measure of the enzymatic activity of the purified PFK1 isoforms based on the reference assay developed by Van Schaftingen et al., Eur. J. Biochem 1982, but while performing the tests we realized that the only commercially available kit to assay for PFK1 activity (Biovision PFK activity colorimetric assay kit), even when testing different batches, has serious problems due to aspecific assay development (the color develops just by assembling the reaction components without any possible PFK source, thus precluding any further measure). This kit is based on the coupling of a chain of enzymatic reactions, and it is known from a long time that this type of assays is subjected to a number of possible disturbances and contaminants (see for example Kruger et al., 1995 where the measurement of PFK1 activity was used as proof-of-principle for such problems). We are sorry for this.

Moreover, the expression of this mutant seems to be lower in Figure 4B compared to WT and could explain the lower pull down of TEAD4.

We now provide a quantification of the immunoprecipitation bands based on the original .tiff files. This panel has been now moved to Fig. 3J. The ratio of TEAD4/PFK1 is lower for all the doses of the PFK1 mutant. Expression levels of the PFK1 isoforms in lysates is now also provided below the Co-IP panels.

In Figure 4G and H, other mechanism of YAP regulation should be included as controls.

We now provide a comparison with latrunculinA treatment, known to inhibit YAP/TAZ activity (Dupont et al., Nature 2011; Wada et al., Development 2011; Zhao et al., Genes&Dev 2012). This is in Fig. 4F.

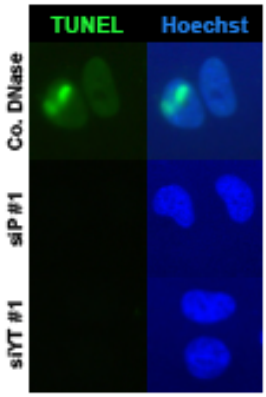
Mutant PFK1 should also be used here.

We tested this but neither wild-type nor mutant PFK1 display any gain-of-function or dominant-negative activity. This is likely due to non-limiting amounts of PFK1 in the cells, or to endogenous feedback mechanisms precisely setting the active subpool of PFK1.

Is the viability of MCF10A-MII cells affected by silencing of PFK1? Could this explain the reduction in mammosphere formation?

In principle yes, it is possible that reduced viability and reduced proliferation concur to define the ability of mammary cancer cells, including MCF10A-MII-TAZ, to grow as mammospheres, even if nobody so far addressed their relative importance. In truth, the precise molecular basis for the growth of mammospheres is largely unknown, even if it is a recognized method to assay for stem cell-related traits. Nonetheless, this would be in any case in line with our data, because both phenotypes (growth arrest and apoptosis) have been described upon glucose inhibition, upon long-term YAP/TAZ silencing (see for example Dupont et al., 2011), and upon placing cells in suspension, which is part of the mammosphere assay. It is interesting to note in this respect that at least part of the effects of cell detachment on cell proliferation and apoptosis is a direct read-out of YAP/TAZ, as shown by Zhao et al., Genes&Dev 2012. We took care in the results and discussion not to confuse the different terms, and to speak about proliferation only when BrdU was directly assessed, while speaking about cell growth and survival in other cases.

To directly answer the Reviewer's question, we performed a TUNEL experiment to compare early signs of apoptosis in MCF10A-MII cells upon depletion of PFK1 or YAP/TAZ but there was no difference with Control siRNA (i.e. we could see no signs of apoptosis). Please find here below a representative panel for this experiment, where DNase treatment served as a positive control (Co.) for the staining. This excluded that reduced viability of PFK1-depleted cells at the moment of mammosphere seeding might false our results (in other words, that we were plating dying or dead cells). This has been included as data not shown in the methods.



How specific is the YAP/TAZ inhibitor used in Figure 5I? How is YAP/TAZ TEAD interaction altered in these cells following different treatments?

The specificity of the inhibitor is unknown. This drug is FDA approved for the photodynamic therapy of macular degeneration (i.e. the drug is inactive until activated by a laser light to kill the surrounding tissue), and it is usually administered to human patients intravenously, so it should have no strong toxic or adverse effects.

In the original publication where it was isolated as YAP/TEAD inhibitor, it was showed that VP treatment of mice by i.p. injection can prevent the development of liver cancer caused by YAP overexpression or NF2/merlin inactivation, and no adverse effect was reported (Liu-Chittenden et al., Genes&Dev 2012). VP has been used since in several publications to inhibit YAP/TAZ activity, both in vitro and in vivo (Feng et al., Cancer Cell 2014; Yu et al., Cancer Cell 2014; Liu et al., Oncogene 2014; Rayon et al., Dev Cell 2014; Imajo et al., NCB 2015). One report indicates that VP may also inhibit autophagy (Donohue et al., JBC 2011), but the underlying mechanism is unknown, and it remains to be determined whether this depends or not on inhibition of YAP/TAZ activity.

We now show in Supplementary Fig. 4E that both 2DG and VP treatment inhibit the binding of YAP to TEAD1 at the endogenous protein level in UOK262 cells.

It would be useful to add indicators of statistical significance into the figure where possible, particularly in Figure 6.

We have now included p values in the figures or figure legends, with particular care for Fig. 6.

Scale bars are missing from the micrographs.

This has been fixed. Thanks.

The text is very difficult to read and the figures need to be labelled more clearly.

We are sorry. We rephrased and simplified where possible, we moved some panels between Fig. 4 and Fig. 3, and revised labeling.

Page numbers need to be added.

This has been now amended. Sorry.

2nd Editorial Decision 19 February 2015

Thank you very much for the revised study.

One of the original referees assessed your revised paper with no further experimental comments and therefore supporting eventual publication in The EMBO Journal.

Please allow me already at this stage to congratulate you to a very insightful study.

