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MIG-6 is essential for promoting glucose metabolic reprogramming and tumor growth in TNBC

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1st Editorial Decision 12th Jun 2020

Dear Dr. Chan,

Thank you for submitting your manuscript to EMBO Reports. Three referees agreed to review your manuscript. So far, we have received two referee reports that are copied below. Given that both referees are in fair agreement that you should be given a chance to revise the manuscript, I would like to ask you to begin revising your study along the lines suggested by the referees.

Please note that this is a preliminary decision made in the interest of time, and that it is subject to change should the third referee offer very strong and convincing reasons for this. As soon as/if we receive the final report on your manuscript, we will forward it to you as well.

My apologies for this unusual delay in getting back to you, it took longer than anticipated to receive the referee reports.

Referees find the proposed role of MIG-6 in TNBC tumorigenesis potentially interesting. However, they also raise concerns that need to be addressed prior to publication here.

I find the reports informed and constructive, and believe that addressing the concerns raised will significantly strengthen the manuscript.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and have therefore extended our 'scooping protection policy' to cover the period required for a full revision to address the experimental issues highlighted in the editorial decision letter. Please contact the scientific editor handling your manuscript to discuss a revision plan should you need additional time, and also if you see a paper with related content published elsewhere.***

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1. A data availability section providing access to data deposited in public databases is missing (where applicable).
2. Your manuscript contains statistics and error bars based on n=2 or on technical replicates.

Please use scatter plots in these cases.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

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1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14693178/authorguide#transparentprocess
You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

4) a complete author checklist, which you can download from our author guidelines (<http://embor.embopress.org/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<http://embor.embopress.org/authorguide>).

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a

short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <http://embor.embopress.org/authorguide#expandedview>.

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) We would also encourage you to include the source data for figure panels that show essential data.

Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available <http://embor.embopress.org/authorguide#sourcedata>.

8) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <http://embor.embopress.org/authorguide#datacitation>.

9) Please make sure to include a Data Availability Section before submitting your revision - if it is not applicable, make a statement that no data were deposited in a public database. Primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see <http://embor.embopress.org/authorguide#dataavailability>).

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

10) Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates.
Please also include scale bars in all microscopy images.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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

Yours sincerely,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

This is a well done manuscript examining the connection between MIG6 and GLUT1. The physiological relevance of the connection is supported by in vivo and in vitro experiments along with clinical data looking at MIG6 and GLUT1 in tumor specimens. The data is convincing and the manuscript is well written. There were a small number of grammatical errors that can be corrected with a careful proofreading. The model is that MIG6 recruits the deubiquitinase HAUSP to HIF1a leading to its stabilization and the subsequent transcriptional upregulation. The connection between HIF1a and GLUT1 is well established so that does not need to be explored in detail here. The mechanistic details of how MIG6 might stabilize HIF1a need to be probed in more depth to make this a complete story.
Major concerns:
1) The mechanistic experiments are conducted in HEK293 cells, making one question the physiological relevance of these findings to TNBC
2) The mechanistic experiments are focused on loss of MIG6, effectively testing its necessity for HIF1a stability. Experiments to test MIG6 sufficiency for HIF1a stability in cells +/- HAUSP would strengthen mechanistic aspects of the model. Likewise, showing that MIG1 expression can induce GLUT1 expression in a manner that depends on HIF1 and HAUSP would also help close the circle on this set of mechanistic experiments.
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model under both normoxic and hypoxic conditions.
4) From the IPs in Fig 5C, the stoichiometry between HIF1a and Myc-HAUSP doesn't make sense. From the model, one predicts a 1:1 interaction. Further in this experiment MIG6 knockdown has a less dramatic effect on HIF1 levels in the absence of HAUSP as compared to the complete dependence shown in figures 5 A and B. One also expects that HAUSP over expression should stabilize HIF1a, again referring to Fig 5C, and that effect would be abrogated in the MIG6 knockdown if the model is correct.

Referee #2:

In their manuscript, He et al. describe a functional link between MIG-6 and glucose metabolism in the very aggressive triple negative breast cancer (TNBC). They report high MIG-6 gene and protein expression in TNBC compared to other breast tumor types, and an association between high MIG-6 protein expression and poor patient survival, inferred from the histopathology analysis of an impressive collection of 85 TNBC tissue samples with patient clinical follow-up. Mechanistically, they found that MIG-6 increases the expression the glucose transporter GLUT1 and other glycolytic genes, which is mediated by HAUSP deubiquitinase recruitment to HIF-1a, leading to HIF-1a stabilisation, enabling glycolytic gene induction. They show that TNBC cell lines proliferate more in presence of MIG-6.
Theses are important fundamental discoveries about MIG-6 in positive regulation of tumor progression, which contrasts with its known role to inhibit tumor cell proliferation by interfering with EGF-R signaling, as the authors indicated. The findings have potential implications for the clinics, and the molecular mechanisms are clearly provided and, in general, convincing. The manuscript is well written. I have a few comments:

Major points:

1) There is a misleading point that requires clarification: in Fig. 1C, the authors show by WB that MIG-6 is strongly expressed in TNBC cells compared to other breast tumor cell lines. However, from Fig. 3A there is a near absence of MIG-6 expression in the TNBC cell line BT549 in normoxia. Instead, MIG-6 becomes visible only under hypoxia + long term exposure. How do the authors explain this discrepancy, which is really confusing? Was the WB of Fig. 1C obtained from cell lines all grown under hypoxia? Additionally, the fact that MIG-6 is induced in hypoxic conditions - which should be confirmed in other cell lines, including TNBC and non-TNBC breast tumor lines - does not add anything to the story at present. I would suggest either to remove Fig. 3A, or to complement it with other cell lines and also using the 85 tumor sections, to test if there is a spatial co-localization between HIF-1a and MIG-6 proteins.

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2) Page 10, end of 1st paragraph, I think the authors wanted to write "GLUT1 gene alteration"

instead of "GLUT1 protein alteration".

3) I agree with the authors' conclusion that "MIG-6 regulates GLUT1 expression and function by promoting transcription of the GLUT1 gene" (page 10). Because the authors possess (because they have used in their study) a V5-tag GLUT1 plasmid, a nice but not essential experiment to confirm that MIG-6 does not interfere with GLUT1 protein would be to show that, in TNBC cells, MIG-6 knockdown does not reduce ectopic GLUT1 protein expression, using an anti-V5 antibody.

4) Because GLUT1 gene transcription is mediated by RNA polymerase II, I would change "is mediated" by "can be enhanced" four lines before the end of page 10.

5) In Fig. 5F, it is strange to see that HIF-1a (don't forget the "1" on the Figure) is well expressed in the input, while shMIG-6 should have decreased it, as seen in Fig. 5B and C. Did the authors use MG132 in this experiment, similarly to Fig. 5G? If yes, please indicate this. If no, please explain why there is no reduction in HIF-1a from the input, in the MIG-6 knockdown conditions reported in Fig. 5F.

6) Fig. 1E: from which comparison was the p-value computed? 3 vs. 2, 3 vs. 1, 3 vs. (1 and 2)? Please indicate, and which statistical test was performed.

7) Fig. 6B: write "Relative lactate" instead of "Lactate" on the y-axis, to fit with Fig. 6A and 3H.

8) The in vivo experiments, unfortunately, only represent what happens already in vitro: shMIG-6 diminishes tumor cell proliferation in vitro, then cells are injected into mice and the shMIG-6 cells continue to proliferate less than control sh. A more sophisticated approach with an shRNA that is induced only in vivo, once tumors are established, would be stronger to enable to conclude that in vivo TNBC tumor growth is affected by MIG-6 knockdown. This would typically rely on TNBC (for example BT549) cell transduction by a lentiviral construct containing TetO-shRNA and rtTA. Doxycycline-mediated shRNA expression would be done in vivo, and the control would be the same transduced cell line without doxycycline addition. However, in the current pandemic it might be logistically difficult or too time consuming to perform this experiment.

Point-by-point response to the reviewers' comments (EMBOR-2020-50781-T)

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This is a well done manuscript examining the connection between MIG6 and GLUT1. The physiological relevance of the connection is supported by in vivo and in vitro experiments along with clinical data looking at MIG6 and GLUT1 in tumor specimens. The data is convincing and the manuscript is well written. There were a small number of grammatical errors that can be corrected with a careful proofreading. The model is that MIG6 recruits the deubiquitinase HAUSP to HIF1a leading to its stabilization and the subsequent transcriptional upregulation. The connection between HIF1a and GLUT1 is well established so that does not need to be explored in detail here. The mechanistic details of how MIG6 might stabilize HIF1a need to be probed in more depth to make this a complete story.

We truly appreciate the reviewer for recognizing the novelty and significance of our work and for providing constructive suggestions. In our point-by-point responses to reviewers below, we have addressed the residual concerns through additional experimental data, text revision, or further discussion. We hope that you will find the current manuscript compelling, exciting, and ready for publication in *EMBO Reports*.

Major concerns:

1) The mechanistic experiments are conducted in HEK293 cells, making one question the physiological relevance of these findings to TNBC

To validate the physiological relevance of our mechanistic experiments, the mechanistic experiment that was conducted in HEK293 cells has now been examined in BT549 cells. Consistent with our previous findings in HEK293 cells, our new result from BT549 cells supports the notion that HAUSP reduces the K48-linked ubiquitination of HIF1α and that this deubiquitination process is mitigated upon MIG-6 knockdown (Figure EV5F).

2) The mechanistic experiments are focused on loss of MIG6, effectively testing its necessity for HIF1a stability. Experiments to test MIG6 sufficiency for HIF1a stability in cells +/- HAUSP would strengthen mechanistic aspects of the model. Likewise, showing that MIG1 expression can induce GLUT1 expression in a manner that depends on HIF1 and HAUSP would also help close the circle on this set of mechanistic experiments.

We thank the reviewer for these constructive suggestions. To further strengthen the mechanistic aspect of our model, i.e., testing MIG-6 sufficiency for HIF1α stability in response to HAUSP expression, we performed two new cycloheximide (CHX) experiments. The first CHX experiment showed that HAUSP knockdown reduces HIF1α stability (Figure EV5G), indicating an essential role of HAUSP in HIF1α stability. Next, we overexpressed MIG-6 in GFP- and HAUSP-knockdown BT549 cells and showed that ectopic expression of MIG-6 increases HIF1α stability in GFP- but not HAUSP-knockdown BT549 cells (Figure EV5H), suggesting that MIG-6-promoted HIF1α stability depends on HAUSP expression.

Additionally, we examined the effects of MIG-6 overexpression on GLUT1 expression in GFP-, HIF1α-, and HAUSP-knockdown BT549 cells. Indeed, our new data demonstrated that MIG-6

overexpression promotes GLUT1 expression and that this effect depends on the expression of HIF1α and HAUSP (Figure 5, I and J).

3) It seems odd that there is so much HIF1a in the control knockdown cells under what are presumably normoxic conditions. What are the growth conditions as HIF1a is generally quite difficult to detect under normoxic conditions? It would also be worth probing the mechanistic details of their model under both normoxic and hypoxic conditions.

We thank the reviewer for this comment. Indeed, the TNBC cells shown in Figure 5 were grown under normoxic conditions. We agree with the reviewer that HIF1α is highly degradable and is often undetectable in many cell models under normoxia. However, Briggs et al. (Cell 2016) reported that HIF1α protein is upregulated in TNBC tissues and showed that multiple TNBC cell models, including those used in our study, express high levels of HIF1α protein under normoxic conditions. Consistent with their finding, we found that TNBC cells such as BT549 and MDA-MB-231 express high levels of HIF1α, while non-TNBC cells such as MCF7 and ZR75-1 express very low levels of HIF1α under normoxia (Figure 5 and Figure EV5A). To further examine the effect of hypoxia on TNBC and non-TNBC cell models, we grew the cells in normoxic and hypoxic conditions. Our new data demonstrate that hypoxia substantially enhances HIF1α protein expression in TNBC and non-TNBC cells (Figure EV5A).

4) From the IPs in Fig 5C, the stoichiometry between HIF1a and Myc-HAUSP doesn't make sense. From the model, one predicts a 1:1 interaction. Further in this experiment MIG6 knockdown has a less dramatic effect on HIF1 levels in the absence of HAUSP as compared to the complete dependence shown in figures 5 A and B. One also expects that HAUSP over expression should stabilize HIF1a, again referring to Fig 5C, and that effect would be abrogated in the MIG6 knockdown if the model is correct.

The IP experiment was shown in Figure 5F in our previously-submitted manuscript, so we assume the reviewer was referring to Figure 5F rather than Figure 5C. We would like to clarify that the IP experiment in Figure 5F was in fact conducted in the presence of MG132 treatment, therefore MIG-6 knockdown did not have much effect on HIF1α downregulation, as shown in Figures 5B and 5C in the revised manuscript. We apologize for missing the description of MG132 treatment in our previous Figure 5F, and for the confusion that it caused. The treatment of MG132 was needed to rescue HIF1α protein degradation mediated by MIG-6 knockdown, leading to similar HIF1α protein levels in control and MIG-6 knockdown BT549 cells, which were used as the input to examine the role of MIG-6 in the interaction between HIF1α and HAUSP. The experimental design with MG132 treatment allowed us to conclude that MIG-6 deficiency mitigates the binding between HAUSP and HIF1α and that the defective interaction between HIF1a and HAUSP was not due to the lower HIF1α expression in MIG-6-knockdown cells (Figure 5F). To avoid the confusion, we have now added a description of MG132 treatment in Figure 5F and in its figure legend (please see page 36). Furthermore, we performed an additional experiment to examine the effects of HAUSP on HIF1α (in the absence of MG132 treatment). Our new data showed that HAUSP overexpression increases HIF1α protein expression and that this effect is mitigated by MIG-6 knockdown (Figure 5K). These findings together underscore that

MIG-6 facilitates HAUSP's interaction with HIF1α, promoting HIF1α protein expression in TNBC cells.

Reference:

[1] Briggs, K. J. et al. Paracrine Induction of HIF by Glutamate in Breast Cancer: EglN1 Senses Cysteine. Cell 166, 126-139, doi:10.1016/j.cell.2016.05.042 (2016).

Referee #2:

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We appreciate the reviewer for recognizing the significance of our study and for his/her constructive comments, which have helped strengthen our manuscript substantially.

Major points:

1) There is a misleading point that requires clarification: in Fig. 1C, the authors show by WB that MIG-6 is strongly expressed in TNBC cells compared to other breast tumor cell lines. However, from Fig. 3A there is a near absence of MIG-6 expression in the TNBC cell line BT549 in normoxia. Instead, MIG-6 becomes visible only under hypoxia + long term exposure. How do the authors explain this discrepancy, which is really confusing? Was the WB of Fig. 1C obtained from cell lines all grown under hypoxia? Additionally, the fact that MIG-6 is induced in hypoxic conditions - which should be confirmed in other cell lines, including TNBC and non-TNBC breast tumor lines - does not add anything to the story at present. I would suggest either to remove Fig. 3A, or to complement it with other cell lines and also using the 85 tumor sections, to test if there is a spatial co-localization between HIF-1a and MIG-6 proteins.

We thank the reviewer for bringing this point to our attention and apologize for the confusion. The TNBC cell lines used in Figure 1C were indeed cultured under normoxia, and we found that hypoxia could further robustly enhances MIG-6 protein expression, as shown in Figure 3A. In this figure, the exposure time was a lot shorter than that which was used for Figure 1C to prevent hypoxia-enhanced saturation of MIG-6 protein signal. We have now provided results of the Western Blot with a longer exposure (L.E.), which confirms the high MIG-6 protein expression in BT549 cells under normoxia (new Figure 3A). Moreover, we performed additional experiments to examine MIG-6 protein expression in TNBC and non-TNBC cell lines under normoxia and hypoxia. Our new data showed that besides BT549, hypoxia induced MIG-6 protein expression in another TNBC cell model, MDA-MB-231, whereas hypoxia did not significantly increase MIG-6 protein expression in non-TNBC cells (Figure EV3A). Additionally, we examined the spatial co-localization between MIG-6 and HIF1α proteins using the 85 TNBC tumor sections. We used the OPAL multiplex IHC assay for simultaneous staining of MIG-6, HIF-1α, and DAPI in each tumor section (n=85). The representative images showed positive immunostaining of MIG-6 and HIF1α proteins in the nucleus and the cytoplasm (Figure 8E). Of

note, MIG-6 and HIF1α were co-localized in both cellular compartments *in vivo*. The triangle indicates the nuclear co-localization; the star indicates the cytoplasmic co-localization (see merged image in Figure 8E). We would like to note that while HIF1α is a transcription factor and its immunostaining primarily is concentrated in the nucleus, cytoplasmic staining of HIF1α has been reported in several human cancers, including breast cancer (Zhong *et al.* Can Res 1999, Tan *et al.* Breast Cancer Res 2007, and Nalwoga *et al.* PLoS ONE 2016). Our finding is consistent with these published studies.

2) For the IP-WB of Fig. 5F, there should be an immunoprecipitation control with another antibody performed. Otherwise we cannot rule out that Myc-HAUSP is sticking to the beads rather than being co-immunoprecipitated.

As suggested, we have now included an immunoprecipitation control with Flag-tag antibody in our IP experiment (new Figure 5F). Our new data show that the Myc-HAUSP was specifically pulled down by HIF1α but not the Flag-tag antibody; moreover, the interaction between HIF1α and HAUSP is mitigated upon MIG-6 knockdown (new Figure 5F). This result supports the reliability of the interaction signal between HIF1α and HAUSP and confirms the notion that the binding between HAUSP and HIF1α depends on MIG-6 in BT549 cells.

Minor points:

1) Fig. S2. To knockdown MIG-6 in PC9 cells, which shRNA was used, #1, #2 or another one?

We thank the reviewer for the comment and apologize for our unclear description. We have now specified that the MIG-6 shRNA#2 was used to knock down MIG-6 in PC9 cells (see Figure EV2A).

2) Page 10, end of 1st paragraph, I think the authors wanted to write "GLUT1 gene alteration" instead of "GLUT1 protein alteration".

We thank the reviewer for the correction. We have revised the description accordingly (page 10).

3) I agree with the authors' conclusion that "MIG-6 regulates GLUT1 expression and function by promoting transcription of the GLUT1 gene" (page 10). Because the authors possess (because they have used in their study) a V5-tag GLUT1 plasmid, a nice but not essential experiment to confirm that MIG-6 does not interfere with GLUT1 protein would be to show that, in TNBC cells, MIG-6 knockdown does not reduce ectopic GLUT1 protein expression, using an anti-V5 antibody.

As suggested, we have now included this experiment using an anti-V5-antibody to probe ectopic GLUT1 protein expression. Our new data confirm that MIG-6 knockdown does not reduce ectopic GLUT1 protein expression (new Figure 6, E and F).

4) Because GLUT1 gene transcription is mediated by RNA polymerase II, I would change "is mediated" by "can be enhanced" four lines before the end of page 10.

The change has been made as suggested (now on page 11 of the revised manuscript).

5) In Fig. 5F, it is strange to see that HIF-1a (don't forget the "1" on the Figure) is well expressed in the input, while shMIG-6 should have decreased it, as seen in Fig. 5B and C. Did the authors use MG132 in this experiment, similarly to Fig. 5G? If yes, please indicate this. If no, please explain why there is no reduction in HIF-1a from the input, in the MIG-6 knockdown conditions reported in Fig. 5F.

We thank the reviewer for bringing this point to our attention and apologize for the confusion. We would like to clarify that the IP experiment in Figure 5F was in fact conducted in the presence of MG132 treatment. The treatment of MG132 was needed to rescue HIF1α protein degradation mediated by MIG-6 knockdown, resulting in similar HIF1α protein levels in control and MIG-6 knockdown BT549 cells, which were used as the input for studying the role of MIG-6 in the interaction between HIF1α and HAUSP. The experimental design allowed us to conclude that MIG-6 is essential for the binding between HAUSP and HIF1α and that the defective interaction between HIF1α and HAUSP was not due to the lower HIF1α expression in MIG-6-knockdown cells. We now have added the description of MG132 treatment in Figure 5F and in its figure legend on page 36.

6) Fig. 1E: from which comparison was the p-value computed? 3 vs. 2, 3 vs. 1, 3 vs. (1 and 2)? Please indicate, and which statistical test was performed.

The *p*-value of 0.011 shown in Figure 1E is used for the comparison of all three groups by Kruskal-Wallis H test. We have now added the description to the figure legend (see page 33). As for the P-values of pairwise comparisons, the *p*-values determined by Mann-Whitney U test are shown as follows: 1 vs. 2=0.202; 2 vs. 3=0.005; and 1 vs. 3=0.006.

7) Fig. 6B: write "Relative lactate" instead of "Lactate" on the y-axis, to fit with Fig. 6A and 3H.

The change has been made as suggested (please see Figure 6B).

8) The in vivo experiments, unfortunately, only represent what happens already in vitro: shMIG-6 diminishes tumor cell proliferation in vitro, then cells are injected into mice and the shMIG-6 cells continue to proliferate less than control sh. A more sophisticated approach with an shRNA that is induced only in vivo, once tumors are established, would be stronger to enable to conclude that in vivo TNBC tumor growth is affected by MIG-6 knockdown. This would typically rely on TNBC (for example BT549) cell transduction by a lentiviral construct containing TetO-shRNA and rtTA. Doxycycline-mediated shRNA expression would be done in vivo, and the control would be the same transduced cell line without doxycycline addition. However, in the current pandemic it might be logistically difficult or too time consuming to perform this experiment.

We appreciate the reviewer's constructive suggestion. As suggested, we established the BT549 stable cells with a lentiviral construct containing the doxycycline-induced MIG-6 shRNA (iMIG-6-shRNA) along with the control BT549 cells with a lentiviral construct containing doxycycline-induced non-targeting shRNA (iNT-shRNA). We first confirmed that doxycycline induced effective MIG-6 knockdown in the iMIG-6-shRNA but not the iNT-shRNA BT549 cells by Western Blot (Figure 7E). These cells were then orthotopically injected to two sides of the nude mice for tumorigenesis assay (Figure 7G). When the tumors reached ~80–100 mm^3, mice were fed

doxycycline chow continuously. Our new data showed that doxycycline-inducible MIG-6 knockdown suppresses the growth of established TNBC *in vivo* (Figure 7, F and G). The new experimental evidence along with our previous data on *in vivo* tumorigenesis assays (now in Figure 7, A–D) collectively underscore an essential role of MIG-6 in tumor initiation and growth in TNBC.

References

Zhong, H. et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 59, 5830-5835 (1999).

Tan, et al. Cytoplasmic location of factor-inhibiting hypoxia-inducible factor is associated with an enhanced hypoxic response and a shorter survival in invasive breast cancer. Breast Cancer Res 9, R89 (2007)

Nalwoga, et al. Strong Expression of Hypoxia-Inducible Factor-1alpha (HIF-1alpha) Is Associated with Axl Expression and Features of Aggressive Tumors in African Breast Cancer. PLoS One 11, e0146823 (2016)

Referee #3:

An interesting manuscript proposing a role for the MIG-6 protein in triple negative breast cancer via ubiquitin-mediated regulation of GLUT1 expression and subsequent effects on glucose metabolism. While there are some interesting findings presented, in general the story is incomplete, the order in which some results are presented does not follow a logical flow, and some mechanistic claims made are not strongly supported by the data.

We appreciate the reviewer for recognizing the significance of our study and for his/her constructive comments, which have helped us to strengthen our manuscript substantially.

1. The title is somewhat misleading, as none of the experiments described within directly address the role of MIG-6 in tumourigenesis. The data describe the dependence of TNBC cells on the continued expression of MIG-6 for proliferation via stimulation of GLUT1 expression and enhanced glucose uptake but these do not necessarily imply a role in tumour formation.

In our study (in both previously performed and additional experiments), we carried out multiple tumor growth assays with stable and inducible cell models to examine the role of MIG-6 in TNBC *in vivo*. Our data demonstrated that genetic targeting of MIG-6 suppressed tumor initiation and growth (Figure 7, A–G), underscoring a cancer-promoting role of MIG-6 in TNBC. We agree with reviewer that these experiments address the role of MIG-6 in tumor growth but not directly on tumor transformation and formation. To avoid potential confusion, we have changed the title to "MIG-6 is essential for promoting glucose metabolic reprogramming and tumor growth in triple-negative breast cancer."

2. There is a mixed logic throughout the manuscript regarding conclusions that can be made about the role of MIG-6 based on knockdown experiments. It is not necessarily the case that the effect of gene knockdown implies the opposite effect of overexpression. This flawed logic is applied numerous times. For example (p8), claiming that MIG-6 "upregulates several glycolytic genes" because expression of these genes is attenuated in MIG-6 shRNA cells, that MIG-6 "downregulated TCA-cycle regulating genes" because these genes are upregulated with MIG-6 shRNA, or "Mig-6s positive regulation of the IDH1/2 gene" is not supported by the data and should be rewritten. Similarly, it is not accurate to state that MIG-6 "promotes" aerobic glycolysis. Rather, MIG-6 knockdown attenuates aerobic glycolysis in TNBC cell lines.

We thank the reviewer for the careful reading of these sentences. We have carefully reviewed all statements in our manuscript and revised them to reflect the implications of our knockdown experiments. For instance, the statement that "MIG-6 upregulates several glycolytic genes" has been corrected to "MIG-6 deficiency inhibits several glycolytic genes"; the statement that "MIG-6 downregulated TCA-cycle regulating genes" has been changed to "MIG-6 deficiency promotes TCA cycle-regulating genes"; the statement that "Our data on MIG-6's positive regulation of the IDH1/2 gene…" has been changed to "Our data that the IDH1/2 genes are downregulated by MIG-6 knockdown…"; and the subheading "MIG-6 promotes aerobic glycolysis" has been changed to "MIG-6 knockdown attenuates aerobic glycolysis".

3. The last paragraph of the introduction is somewhat pre-emptive of the data following and could be significantly shortened.

As suggested, the last paragraph of the introduction has been shortened (page 4).

4. Figure1F/G: How was "high" and "low" MIG-6 expression determined? Was expression across tumours in the cohort normally distributed?

We thank the reviewer for the questions. To answer these questions, we have now provided more detailed information in the "Immunohistochemistry and scoring" section of the Materials and Methods, as follows: MIG-6 protein expression in the 85 TNBC specimens was determined by H-score, which was generated based on a combination of both the percentage and intensity of positively stained tumor cells and calculated using the following equation: H-score = ΣPi (i + 1), where i is the intensity of the stained tumor cells (0 to 4+) and Pi is the percentage of stained tumor cells for each intensity. MIG-6 expression greater than or equal to the median is classified as "high," while expression less than the median is classified as "low," as indicated in Table EV2" (please see pages 25-26). To clarify this point, we have also added the above description to the figure legend (please see page 33). Additionally, we have now provided the protein expression data of MIG-6 across the 85 TNBC specimens. Our data showed that MIG-6 expression is widely distributed across the tumors (Figure EV1C). This wide distribution allowed us to analyze the relationship between MIG-6 expression and survival outcomes in TNBC patients (Figure 1, F and G, and Table EV2). One might expect a biomarker to be normally distributed across human tissues. However, cancer is a highly heterogeneous group of diseases and tumor growth relies on various pathways, so it is very unlikely for a biomarker to be normally distributed across all tumor tissues.

5. Insufficient detail is provided about how shRNA experiments were performed. Do these represent cellular pools or selected clones? How long post infection are these analyses performed? Are replicates independent infections or technical replicates using the same pools of cells?

We thank the reviewer for bringing these issues to our attention and apologize for the insufficient information. The experimental details for lentiviral shRNA-mediated gene knockdown experiment are now provided in the revised manuscript (please see pages 19–20 for details). In brief, the stable cell lines represent cellular pools after puromycin selection for at least four days. Every time the knockdown efficiency of MIG-6 was confirmed by immunoblotting, the stable cells with successful knockdown were used in various analyses, as shown in the current study. Each batch of control and MIG-6-knockdown stable cell lines was used for 2–3 months, then replaced with newly generated stable cells. While the quantitative results were generated by technical replicates, all the phenotypes were verified by at least two different batches of stable cell lines.

6. Figure2c: should be shown as actual colony number, not relative percentage.

The actual colony number is now shown in Figure 2C, as suggested.

7. The link between data in Fig2 and the subsequent decision to pursue a role for MIG6 in glycolysis is not very clear and could be better justified. Do EGFR inhibitors have any effect on cell proliferation/metabolism with MIG-6 knockdown in TNBC cells?

We thank the reviewer for the insightful question. We have now performed additional experiments to elucidate the effects of the EGFR inhibitor gefitinib on the survival and metabolism of GFP- and MIG-6-knockdown BT549 cells. Our new data showed that gefitinib suppresses the growth of BT549 cells in a dose-dependent manner; moreover, MIG-6 knockdown further suppresses cell growth under gefitinib treatment (Figure 2F). Additionally, we showed that gefitinib inhibited lactate production in BT549 cells and that MIG-6 knockdown further enhances the inhibitory effect (Figure EV3G). These results suggest that MIG-6 regulates an EGFR-independent mechanism (such as GLUT1 upregulation, as characterized in the current study) to promote the growth and metabolism in TNBC cells.

8. Figure 4A: Are MIG-6 and GLUT1 mRNA expression elevated in the same individual tumours? Any correlation should apparent using an x-y plot of MIG-6 vs GLUT1 expression.

We thank the reviewer for this interesting point. While we showed in our previously submitted manuscript that MIG-6 protein level is significantly correlated with GLUT1 protein expression in TNBC tumor specimens (now in Figure 8, C and D, and Table EV1), it is interesting to probe whether the correlation occurs at the mRNA level. To address this question, we utilized the R2 bioinformatics tool to retrieve two transcriptome datasets [Servant *et al.* (GSE30682) and Bertucci *et al.* (GSE21653)] (see Figure 4A and Figure EV4A), then examined the correlation between MIG-6 and GLUT1 mRNA levels in these datasets. Our data showed that MIG-6 is positively correlated with GLUT1 at the mRNA level in basal-like breast cancer/TNBC in both datasets (Appendix Figure S1, A and B) although only the result from the Bertucci dataset has statistical significance (Appendix Figure S1A).

9. Figure4: It is not clear which band(s) represent GLUT1 in western blots shown in panels C and F.

GLUT1 (encoded by the SLC2A1 gene) is a membrane glycoprotein. Glycosylation of GLUT1 is known to play an important role in maintaining high-affinity transport for glucose[1]. The molecular weight of unglycosylated GLUT1 is about 54–55 kDa. The glycosylation of GLUT1 leads to a smeared banding pattern[1], as shown in previous reports[2,3] and the datasheet below. The degree of GLUT1 glycosylation is known to differ in cell types[4], as does the smeared pattern. The Western Blot below shows a smeared banding pattern of GLUT1 in A549 lung cancer cells, and the smeared pattern disappeared in GLUT1-knockout (KO) A549 cells, supporting the notion that the smeared banding represents modifications of the GLUT1 protein. In line with the observations, the smeared pattern was observed in both endogenous and exogenous GLUT1 (Figure 4, C and F, and Figure 6, E and F) in TNBC cells. Of note, the smeared pattern was also detected by the V5-tag antibody (Figure 6, E and F), indicating that the smeared pattern comes from GLUT1 protein moiety.

10. Figure 4E: Gates/regions for quantifying 2-NBDG uptake are not aligned between control and shMIG6 samples.

We apologized for this careless mistake. We re-reviewed the gating criteria in our original analysis and redrew the gate/regions for quantifying 2-NBDG accordingly. The gates/regions have now been aligned between the control and shMIG-6 samples (Figure 4E).

11. Figure5: An important control is missing in Panel E, effect of MG132 in shGFP cells. Further, the decreased detection of Myc-HAUSP following HIF1a IP in shMIG-6 cells is not clear from this experiment as there appears to be significantly different exposure/background levels in the relevant regions of this blot. Data should be shown supporting the statement that MIG-6 did not affect formation of the VHL/HIF1a complex.

We thank the reviewer for the comments. We have now repeated the experiment presented in Figure 5E and have included a control to determine the effect of MG132 on shGFP cells. As expected, our new data showed that MG132 stabilizes HIF1α expression in shGFP cells (Figure EV5D). We would like to note that because MG132 treatment robustly stabilizes HIF1α, we intentionally kept an empty well between the samples with and without MG132 treatment to prevent signal interference from the MG132-treated samples.

Additionally, we repeated the HIF1α IP experiment. Our new data reproducibly showed that MIG-6 knockdown reduces the interaction between HIF1α and Myc-HAUSP (Figure 5F).

We have also now presented the data in Figure EV5E, as suggested. The data showed that MIG-6 deficiency did not enhance formation of the VHL/HIF1α complex, suggesting that VHL-mediated protein degradation of HIF1α does not contribute to HIF1α downregulation by MIG-6 knockdown.

12. Figure5G: Control missing (i.e shMIG6 cells without His-Ub). Also, this experiment uses a highly artificial system relying on expression of multiple tagged exogenous proteins. It would be preferable to show some similar effect on endogenous proteins.

We thank the reviewer for the comment. We have repeated the experiment with the suggested control (i.e., without His-Ub) for shMIG-6 cells and examined the ubiquitination level of endogenous instead of exogenous HIF1α (Figure EV5F). While, as expected, the overall ubiquitination signal of endogenous HIF1α is weaker than that of exogenous HIF1α (as shown in Figure 5G), our new data consistently showed that HAUSP reduced the K48-linked ubiquitination of HIF1α and that this deubiquitination process is mitigated upon MIG-6 knockdown (Figure EV5F).

13. Figure6: experiments shown in panels E-H are missing an important control. What is the effect of GLUT1 overexpression in shGFP cells?

We have now included a control to examine the effect of GLUT1 overexpression on lactate production in shGFP cells, as suggested. As expected, our new data showed that GLUT1 overexpression enhanced lactate production in both BT549-shGFP and MDA-MB-231-shGFP cells (new Figure 6, E–H).

14. Figure7: Are the metabolic effects of MIG-6 knockdown observed in vitro also apparent in xenografts?

Our *in vitro* studies showed that MIG-6 regulates glycolysis and lactate production through GLUT1, a gatekeeper of glucose uptake and subsequent glycolysis (Figure 4C and Figure 6, E–H). In support of our *in vitro* observations, we performed IHC staining for *in vivo* GLUT1 expression in xenograft tumors and showed that MIG-6 knockdown decreased GLUT1 protein expression in BT549-derived xenograft tumors (Figure 8A). In addition, we examined *in vivo* GLUT1 expression in BT549 xenograft tumors with an inducible MIG-6 knockdown system. Consistently, we found that *in vivo* GLUT1 expression is also decreased in doxycycline-induced MIG-6 knockdown tumors (Figure 8B).

15. Discussion: It is not correct to assert that "MIG-6 is crucial for elevated glucose metabolism and tumorigenesis in TNBC" as no comparison was made to either normal mammary epithelial cells or breast cancer cells of other subtype. This framing should be rewritten to be consistent with the observation that cell proliferation and glycolysis is attenuated with MIG-6 knockdown in two TNBC cell lines. Similarly, the absence of an effect of MIG-6 knockdown on EGFR signaling does not necessarily imply that "MIG-6 fails to inhibit EGFR signaling in TNBC" as this experimental comparison was not made.

We thank the reviewer for the corrections. The statement that "MIG-6 is crucial for elevated glucose metabolism and tumorigenesis in TNBC" has been changed to "MIG-6 depletion attenuates glucose metabolism and tumor growth in TNBC" (please see page 16). Additionally, the statement that "MIG-6 fails to inhibit EGFR signaling in TNBC" has been changed to "MIG-6 depletion does not promote EGFR signaling in TNBC" (please see page 16).

Discussion of the effect of MIG-6 on Y394 phosphorylation is speculative and not supported by data.

A previous study by Park *et al.* demonstrated that Y394 phosphorylation of MIG-6 leads to structural rearrangement that blocks the binding of MIG-6 to EGFR and that MIG-6 phosphorylation at Y394 is essential for MIG-6's effect on EGFR inhibition (Park *et al.,* Nat. Struc. Mol Biol. 2015)[5]. Since our data showed MIG-6 knockdown in TNBC cells failed to promote EGFR signaling (Figure 2, D and E), we speculated that MIG-6 Y394 phosphorylation is defective in TNBC cells. To elucidate this notion, we requested the phosphospecific antibody against the dual pY394pY395 site (denoted as pMIG6 Y394/Y395) from Drs. Park and Cho. Consistent with the finding by Park *et al.*, we found that MIG-6 phosphorylation at Y394/Y395 is substantiated in the lung cancer cell lines PC9 and H3255 and that the MIG-6 tyrosine phosphorylation is attenuated by the EGFR inhibitor gefitinib (this is because activated EGFR is known to drive the Y394 phosphorylation of MIG-6). Intriguingly, MIG-6 in TNBC cells, albeit expressed at high levels, did not undergo Y394/Y395 tyrosine phosphorylation (Appendix Figure S2). Therefore, we reasoned that the defective Y394/Y395 phosphorylation of MIG-6 in TNBC cells may be one of the mechanisms that attenuates MIG-6's tumor-suppressive effect on EGFR inhibition. To avoid potential overstatement, we have also added the statement that "future studies are needed to verify this notion" in the Discussion section (please see page 17).

16. Discussion at the bottom of p16 overstates experimental findings on the mechanism of MIG-6s role in HAUSP/HIF1a biochemistry. Further, the claim that "MIG-6 comprehensively regulates glucose metabolism" is hyperbolic and could be toned down.

We thank the reviewer for the suggestion. We would like to note that the discussion at the bottom of page 16 in our previously-submitted manuscript (now on pages 17-18 of the revised manuscript) was not based merely on our mechanistic findings on the role of MIG-6 in HAUSP/HIF1a biochemistry, but also on our metabolic array data. These metabolic array data also support the notion that MIG-6 recapitulates HIF1α's role in regulating the expression of several metabolic genes, including GLUT1, HK2, PFK1, ENO2, PGK1, and LDHA, for promoting glycolysis, as well as the role of PDK1 in suppressing oxidative phosphorylation. Nevertheless, the statement that "MIG-6 comprehensively regulates glucose metabolism" has been amended to "MIG-6 exhibits pleiotropic effects on glucose metabolism" to avoid overstatement (please see page 18).

References:

1. Mora S, Pessin J. Glucose/Sugar Transport in Mammals. *Encyclopedia of Biological Chemistry.* page 391-394 (2013)
2. Ferrer, C. M. *et al.* O-GlcNAcylation regulates cancer metabolism and survival stress signaling via regulation of the HIF-1 pathway. *Mol Cell* **54**, 820-831, doi:10.1016/j.molcel.2014.04.026 (2014).
3. Zhou, L. & Yang, H. The von Hippel-Lindau tumor suppressor protein promotes c-Cbl-independent poly-ubiquitylation and degradation of the activated EGFR. *PLoS One* **6**, e23936, doi:10.1371/journal.pone.0023936 (2011).

4. Kitagawa, T., Tsuruhara, Y., Hayashi, M., Endo, T. & Stanbridge, E. J. A tumor-associated glycosylation change in the glucose transporter GLUT1 controlled by tumor suppressor function in human cell hybrids. *J Cell Sci* **108 (Pt 12)**, 3735-3743 (1995).

5. Park E, Kim N, Ficarro SB, Zhang Y, Lee BI, Cho A, et al. Structure and mechanism of activity-based inhibition of the EGF receptor by Mig6. *Nat Struct Mol Biol.* **22**:703-11 (2015).

1st Revision - Editorial Decision 11th Jan 2021

Dear Lori,

Thank you for submitting your revised manuscript. It has now been seen by two of the original referees.

As you can see, the referees find that the study is significantly improved during revision and recommend publication. Before I can accept the manuscript, I need you to address some minor points below:

• Please address the remaining minor concerns of the referees.
• Please provide 3-5 keywords for your study. These will be visible in the html version of the paper and on PubMed and will help increase the discoverability of your work.
• Please upload Tables EV1-EV4 as individual files.
• Please upload The Appendix figures as individual files. Please add a Table of Contents.
• Please add a sentence to the Data Availability Section stating that you have not deposited any primary data to a depository. Moreover, we note that GSE30682 and GSE21653 have been deposited at GEO, not at Genomic Spatial Event (GSE) as mentioned in this section.
• Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.
• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

This revision addresses the majority of my concerns. There are a few minor points that shold be addressed editorially. Also there are still several grammatical errors that can be corrected with

careful proof reading and line editing.

The last paragraph of introduction is really just a summary of the paper with much of the information in the abstract. This could be shortened.
For Figs 2D and E, please change the text in the results to indicate that MIG6 knockdown didn't change EGFR signaling in response to EGF stimulation.
For figure 3A indicate in the results section the cell line being used, rather than just stating TNBC cells.
Figure 3D and E would be easier to understand they visually reported the decrease in expression of glycolytic gene expression (D) or the increase in OxPhos gene expression (E).
MIG6 knockdown reduced the amount of GLUT1 on the cell surface. The authors conclude that this is due to "Glut1 gene alternation". I believe they mean a reduction in Glut1 gene expression.
It would be useful to show that GLUT1 overexpression corrects the loss of glucose uptake that results from MIG6 knockdown. Never mind, those experiments are in figure 6.
For Figure 7F the modified tumor cells were injected into the flanks of immunocompromised mice and not the mammary fat pad. The description in the results indicated that this an orthotopic model. Please clarify.

Referee #2:

The authors have addressed my comments satisfactorily. As single exception, in response to my first major comment, the authors have used multiplex IF to stain MIG6 and HIF1 from their 85 tumor tissue samples, but have unfortunately not exploited their results, as they only provide a representative staining showing that both proteins can co-localize (Figure 8E). I would encourage the authors to analyze their data and quantify protein expression across the 85 tumor samples. By co-localization, I meant spatial co-localization within tumors areas, not intracellular co-localization.

In short: within tumors, is there a MIG6 and HIF1 expression heterogeneity, with tumor areas where expression is high (or low) for both proteins? In contrast, are there fewer tumor areas expressing one protein but not the other?

Response to the editor's comments (EMBOR-2020-50781-V2)

• Please address the remaining minor concerns of the referees.

- Please see our responses listed below

• Please provide 3-5 keywords for your study. These will be visible in the html version of the paper and on PubMed and will help increase the discoverability of your work.

- Keywords: Glucose metabolism; MIG-6; HIF1α; GLUT1; triple-negative breast cancer

• Please upload Tables EV1-EV4 as individual files.

- We have uploaded the individual files.

• Please upload The Appendix figures as individual files. Please add a Table of Contents.

- We have uploaded the individual files and provided the information accordingly.

• Please add a sentence to the Data Availability Section stating that you have not deposited any primary data to a depository. Moreover, we note that GSE30682 and GSE21653 have been deposited at GEO, not at Genomic Spatial Event (GSE) as mentioned in this section.

- We have added the statement and made the correction accordingly.

• Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.

- Synopsis: Unlike the tumor-suppressive role of MIG-6 in lung tumors, MIG-6 is upregulated in TNBC and is essential for TNBC growth by promoting glucose metabolism via the HIf1α-Glut1 pathway.

- Bullet points:

- ***MIG-6 is a novel metabolism driver that regulates glucose metabolism reprogramming toward glycolysis***
- ***MIG-6 regulates glucose metabolism by promoting GLUT1 gene transcription***
- ***MIG-6 recruits HAUSP deubiquitinase for stabilizing HIF1α protein expression and the subsequent upregulation of GLUT1***
- ***The MIG-6-GLUT1 pathway is critical for tumor growth in TNBC***

• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

- The image has been uploaded.

• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

- The changes have been incorporated (see the newly-uploaded manuscript).

Referee #1:

This revision addresses the majority of my concerns. There are a few minor points that should be addressed editorially. Also there are still several grammatical errors that can be corrected with careful proof reading and line editing.

- The manuscript has been proofread by a professional language editing service.

The last paragraph of introduction is really just a summary of the paper with much of the information in the abstract. This could be shortened.

- As suggested, we further shortened the paragraph from 978 to 482 characters (please see page 4 of the newly-uploaded manuscript).

For Figs 2D and E, please change the text in the results to indicate that MIG6 knockdown didn't change EGFR signaling in response to EGF stimulation.

- The change has been made as suggested (see page 6).

For figure 3A indicate in the results section the cell line being used, rather than just stating TNBC cells.

- The change has been made as suggested (see page 7).

Figure 3D and E would be easier to understand they visually reported the decrease in expression of glycolytic gene expression (D) or the increase in OxPhos gene expression (E).

- Presentation of the data in Figs 3D and 3E is associated with the data presented in Figs 3C-3H, Fig EV3E, and Table EV3. In the current form, the percentage of change in gene dysregulation between the shGFP and shMIG-6 groups is normalized to the shMIG-6 group. The reviewer prefers the normalization to the shGFP group. Essentially both ways are correct and widely used in the literature. We had discussed which way to be used before and the authors decided to use the normalization to shMIG-6 as we believe it would be more intuitive and easy to follow by readers.

MIG6 knockdown reduced the amount of GLUT1 on the cell surface. The authors conclude that this is due to "Glut1 gene alternation". I believe they mean a reduction in Glut1 gene expression.
It would be useful to show that GLUT1 overexpression corrects the loss of glucose uptake that results from MIG6 knockdown. Never mind, those experiments are in figure 6.

- As suggested, the description that “Glut1 gene alteration” has been changed to “a reduction in Glut1 gene expression” (see page 9).

- Indeed, the experiments were done in Figure 6 as the reviewer indicated.

For Figure 7F the modified tumor cells were injected into the flanks of immunocompromised mice and not the mammary fat pad. The description in the results indicated that this an orthotopic model. Please clarify.

- For Figure 7F, the tumor cells were injected into two sides of <u>mammary glands</u> (but not flanks), so it is correct to indicate that this is an orthotopic model. To clarify, we added "mammary glands" to the original statement (see page 12).

Referee #2:

The authors have addressed my comments satisfactorily. As single exception, in response to my first major comment, the authors have used multiplex IF to stain MIG6 and HIF1 from their 85 tumor tissue samples, but have unfortunately not exploited their results, as they only provide a representative staining showing that both proteins can co-localize (Figure 8E). I would encourage the authors to analyze their data and quantify protein expression across the 85 tumor samples. By co-localization, I meant spatial co-localization within tumor areas, not intracellular co-localization.

In short: within tumors, is there a MIG6 and HIF1 expression heterogeneity, with tumor areas where expression is high (or low) for both proteins? In contrast, are there fewer tumor areas expressing one protein but not the other?

- We thank the reviewer for the suggestions. The quantitative results have been shown in Appendix Figure S2. The data indicate that most TNBC tumors exhibit strong colocalization between MIG-6 and HIF1α protein expression.

2nd Revision - Editorial Decision 5th Feb 2021

Dear Lori,

Thank you for sending your revised manuscript. I have now looked at everything carefully and all is fine. Therefore, I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice study!

Kind regards,

Deniz
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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

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

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

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

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?	Sample size is described in each figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size is described in each figure legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
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.	Mice were randomized prior to treatment (see page 24-25 of main text)
For animal studies, include a statement about randomization even if no randomization was used.	The statement is described in section of "Animal studies" on page 24-25 of main text.
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.	Blinding was not done during tumor inoculation but during the measurement of tumor size.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The statement is described in section of "Animal studies" on page 24-25 of main text.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	Yes, the evariation was presented as ±SD or ±SEM within each group of data as described in the figure legends.

Is the variance similar between the groups that are being statistically compared?	Yes, the statistical plan is described on page 26-27 of main text.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The source of antibodies is reported in Table EV4 of the Expanded View Table file.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The information is described on page 19 of main text.

* 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.	Reported in the section of "Animal studies" on pages 24-25 of the main text.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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.	We confirm compliance.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Patient tissues used in this study were obtained with consent under the protocol approved by the institutional review board of the Chi-Mei Foundational Medical Center
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.	The statement is described on page 26 of main text.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The section of "Data availability" is described on page 26 of main text.
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).	N/A
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).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

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