EMBO reports

ZBTB2 links p53-deficiency to HIF-1-mediated hypoxia signaling to promote cancer aggressiveness

Sho Koyasu, Shoichiro Horita, Keisuke Saito, Minoru Kobayashi, Hiroshi Ishikita, Christalle Chow, Gouki Kambe, Shigeto Nishikawa, Toshi Menju, Akiyo Morinibu, Yasushi Okochi, Yoshiaki Tabuchi, Yasuhito Onodera, Norihiko Takeda, Hiroshi Date, Gregg L. Semenza, Ester Hammond, and Hiroshi Harada
DOI: 10.15252/embr.202154042

Corresponding author(s): Hiroshi Harada (harada.hiroshi.5e@kyoto-u.ac.jp)

Review Timeline:

Submission Date:	22nd Sep 21
Editorial Decision:	5th Nov 21
Revision Received:	27th Aug 22
Editorial Decision:	4th Oct 22
Revision Received:	16th Oct 22
Accepted:	19th Oct 22

Editor: Deniz Senyilmaz-Tiebe/Achim Breiling

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **5th Nov 2021**

Dear Dr. Harada,

Thank you for the submission of your research manuscript to our journal, which was now seen by three referees, whose reports are copied below.

We concur with the referees that the proposed role of ZBTB2 in activation of HIF-1 signaling by p53 deficiency in principle very interesting. However, referees raise significant and largely overlapping concerns that need to be addressed to consider publication here.

I find the reports informed and constructive, and believe that addressing the concerns raised will significantly strengthen the manuscript. As the reports are below, and I think all points need to be addressed, I will not detail them here.

Should you be able to address all referee concerns satisfactorily, 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.

*** Temporary update to EMBO Press scooping protection policy:
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.***

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
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. Please use scatter plots in these cases.

You can submit the revision either as a Scientific Report or as a Research Article. For Scientific Reports, the revised manuscript can contain up to 5 main figures and 5 Expanded View figures. If the revision leads to a manuscript with more than 5 main figures it will be published as a Research Article. In this case the Results and Discussion section should be separate. If a Scientific Report is submitted, these sections have to be combined. This will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. In either case, all materials and methods should be included in the main manuscript file

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.

Please note that for all articles published beginning 1 July 2020, the EMBO Reports reference style will change to the Harvard style for all article types. Details and examples are provided at
https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

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.

Kind regards,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Scientific Editor
EMBO Reports

Referee #1:

This manuscript by KOYASU et al. investigated a novel molecular mechanism linking p53 deficiency with HIF-1 activation. By genetic screening, they identified ZBTB2 as an important molecular mediator and critical for invasion and tumorigenesis when p53 is deficient. In addition, the authors mapped the subdomain of ZBTB2 and revealed that its dimerization via N-terminus could mediate the transactivation of HIF-1a, therefore, suggesting a potential therapeutic implication by targeting the ZBTB2-HIF-1 axis in p53 deficient context. Overall, this is a well-designed and presented a study with some novel findings which would be interesting to the cancer research community related to p53 and HIF. However, this work also has a few limitations such as lack of proof-of-concept evidence of ZBTB2 could serve as a therapeutic target either in vitro or in vivo. Specific comments from the reviewer are below.
Major comments
1. Is ZBTB2 the only candidates that activate HIF-1 under P53-deficiency? The authors performed a two-step screening, and from the first-round screening, they identified 4 candidates. However, whether these 4 genes or others were subjected to the second round of screening is not clear. From the description from Line7-9, the authors not stated clearly what genes were used for the second round of screening, and related data was not presented. This indicates the rationale to choose ZBTB2 as the final candidate for further investigation.
2. From the data from Fig. 1A, it seems like the HIF-1 activity elevated, instead of decreased, in the p53 functional (p53+/+) HCT116 cell versus p53-null (p53-/-) under hypoxia, which is not consistent with the hypothesis and initial data from U2OS (Fig. S1). How to explain this discrepancy? Besides, how about the HIF-1a protein level in these conditions?
3. Authors performed lots of gain-of-function by overexpression of ZBTB2 to confirm its oncogenic function in the p53 null content both in vitro and in vivo. However, from the therapeutic perspective of view, whether ZBTB2 is a potential target in the context of p53-deficient is unclear. One will be interested to see the consequence of cell proliferation and tumorigenesis after ZBTB2 genetic depletion either in vitro or in vivo.
4. The authors claimed that the homodimerization of ZBTB2 via its N terminus region increases the transactivation activity of HIF-1α. However, the causal relationship between ZBTB2 homodimerization and transactivation activity of HIF-1α is obscure. In other words, the current data could not fully support the conclusion that the N-terminal of ZBTB2, which is essential for its dimerization, is exclusively critical for HIF-1a transactivation activity. As the data from Fig. 3F clearly showed that other domains including BTB/POZ, ZF2, and ZF3 are also important and caused identical TAD activity loss as the N-terminus region. How to explain this phenomenon? Does the other domain also responsible for the dimerization? Either providing more direct evidence to claim or tune down the conclusion would be helpful.
Minor comments
1. p53 overexpression in Fig. 1C is suggested to confirm either by western blotting or qRT-PCR.
2. The results from Page5, Line24-27, and Page6, Line1-5 does not support the conclusion that ZBTB2 activate HIF-1a, but more fit to the section "Homodimerization of ZBTB2 via its N terminus region increases the transactivation activity of HIF-1α"
3. Please show representative images for the invasion assays from Fig. 2D and 2E.
4. The label is missing for the tumor growth showed in Fig. 2H.

5. In the patient survival analysis from Fig. 2J and K, what's the percentage of p53 deficiency/loss-of-function mutation in these patients? Does the tissue microarray or colorectal cancer cohort contain information of p53 state? If so, it may be helpful to stratify the patient according to p53 state and re-examine the Kaplan-Meier analysis in these patients.

Referee #2:

Koyasu et al. provide evidence that ZBTB2 is linked to HIF1a activation. This is supported by clinical correlation. Figures 1, 2, and 3 are well designed and provide compelling evidence for ZBTB2 and HIF1 activation.

A minor concern in Fig.3b is in HCT116 with ZBTB2 under "normoxia" and hypoxia. It appears to be a significant induction compared to EV. The authors should consider conducting statistical analysis on this data. The authors use less than 0.1% oxygen which is anoxic. The authors should either conduct the experiments under hypoxic 1% or label the text as anoxic.

The authors show ZBTB2 will dimerize with itself in overexpression experiments. However, it is unclear what the physiological condition would be to trigger the dimerization. The authors should consider determining if the dimerization is evident under hypoxia (1% oxygen). The dimerization may be specific to different conditions.

The authors measure tumor growth by volume over time which is important. However, the final tumor weight, examination of necrosis of the tumors, vascularization (new blood vessel formation vs. existing blood vessels), circulating tumor cells, and metastasis would help provide a physiological pathway that ZBTB2 is affecting HIF1 can influence many of these pathophysiological conditions.

It is unclear if the statement that wild type p53 can inactivate HIF1 in the context of nutlin. There are several reports that Mdm2 is necessary for HIF1 activity, and Nutlin will block the Mdm2-HIF1a interaction. Thus, the authors did not address if Nutlin influenced the p53-Mdm2 or Mdm2-HIF1 interaction. Considering this is outside the paper's focus, the authors should consider removing this data or provide a more comprehensive review of the literature in the discussion outlining the current understanding of the HIF1-Mdm2 and p53-mdm2 pathways under hypoxic conditions.

Referee #3:

In this manuscript the authors have tried to bridge the molecular players that are involved between HIF-1-dependent hypoxia signaling and p53-deficiency. They claim to identify ZBTB2, a zinc finger DNA binding protein to be a key mediator in that pathway. Though the study may be interesting, the reviewer's enthusiasm is dampened due to the moderate impact of the study, as well as the study's flawed study design (over dependence on over expression system).

Major criticisms:
1. The authors have not established a direct correlation between the p53 deficiency and induction of HIF1A activity. In fact, there are studies that suggest that HIF1A stabilizes p53 activity (An et al, Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha; Nature 1998; 392(6674):405-8). It is thus counter intuitive, but not impossible, that loss of p53 would induce higher HIF1A activity, but the authors need to show that a significant correlation exist between these two.
2. The screening that identified ZBTB2, along with UCHL1, IDH3a and LY6E as the candidates that can significantly upregulated HIF-1 activity was carried out in HeLa cells, which itself do not have any p53 activity (Figure S2). Thus, it looks like that the authors wanted to put a thumb on the scale here (choosing to use a p53 deficient cell line to identify the candidates) and then making a case that ZBTB2 links p53 deficiency to HIF1A activation. In other words, the screen is not agnostic to the p53 status. It is not a major flaw, but the authors should disclose it right away that they were validating their candidates in a p53 compromised cell lines. This makes the Figure 1 redundant, or at best, the Figure 1 can be added to the Figure S2, to make the case that ZBTB2 increases HIF1A activity in p53 deficient isogenic cell lines. Otherwise, the logic is circular in nature, and the reviewer does not see its significance to merit a main Figure in the manuscript.
3. A major deficiency in the manuscript is that the authors have not established whether ZBTB2-HIF-1 axis is a major driver of aggressive tumor invasion. The studies that have been presented are to make a case of for the said axis to contribute to modest (though significant) increase in tumor invasion/proliferation. But the impact of the study is dampened by the failure of the authors to establish that this axis is necessary and sufficient to maintain and progress tumorigenesis. So, though the study may illuminate that ZBTB2-HIF1A is modulated by dimerization of ZBTB2, the significance of the observation is academic at best. The study is further complicated by the fact that ZBTB2 is ectopically over expressed. It would have been more interesting if the authors could identify cell lines that have high endogenous ZBTB2 expression, with concomitant high HIF1A activity, and carried out the loss of function assays in those cell lines.
4. The Kaplan Meier analysis shown in 2J and 2K correlating the high expression of ZBTB2 with relatively poor outcome is over simplistic. To support their claim, the authors need to find out the p53 and the HIF1A status in the samples as well. Furthermore, the tumors in the TMA are expected to have significant tumor heterogeneity. It is interesting to note that the increased staining

ZBTB2 in the tissue microarray are not necessarily in the invading front? Regardless, it should be doable to find out the p53 status of the cells staining strongly for the ZBTB2, as well as their HIF1A expression.
5. The study's overall dependence on overexpression of ZBTB2 is a major problem. Ectopic over expression of ZBTB2 is most likely not representative of the physiological ZBTB2 levels in the cancer cells. Also, the study depends highly on the assays derived from the HRE reporter systems. Is there a reason that the authors did not opt for a more agnostic approach, for example, transcriptomics analysis?
6. The biochemical assays showing that the homodimerization of ZBTB2 is required for transactivation of HIF1A again relies extensively on the over expression systems. The reviewer opines that a cleaner system is required to derive the conclusions stated, either by engineering of the endogenous ZBTB2 loci with the stated modifications, or by using cell lines where the endogenous ZBTB2 is inactivated. Overall, the study design is problematic, since it overwhelmingly depends on an overexpression system.

Reviewers' comments are in **bold** type and surrounded by a box. Our responses are shown in red.

Reviewer #1:

Overall. This manuscript by KOYASU et al. investigated a novel molecular mechanism linking p53 deficiency with HIF-1 activation. By genetic screening, they identified ZBTB2 as an important molecular mediator and critical for invasion and tumorigenesis when p53 is deficient. In addition, the authors mapped the subdomain of ZBTB2 and revealed that its dimerization via N-terminus could mediate the transactivation of HIF-1a, therefore, suggesting a potential therapeutic implication by targeting the ZBTB2-HIF-1 axis in p53 deficient context. Overall, this is a well-designed and presented a study with some novel findings which would be interesting to the cancer research community related to p53 and HIF. However, this work also has a few limitations such as lack of proof-of-concept evidence of ZBTB2 could serve as a therapeutic target either in vitro or in vivo. Specific comments from the reviewer are below.

Thanks to this comment, we realized that Proof-of-Concept (POC) evidence was insufficient in our original manuscript. Therefore, we carried out additional *in vitro* and *in vivo* POC loss-of-function studies using the siRNA technique and inhibitory polypeptide for ZBTB2 homodimerization (Figs 2B, 2E, 2I, 2G, and 6D-I). In addition, we discussed the need to further conduct POC studies in the future in Discussion of our revised manuscript (Page 13, lines 15-19).

Major comments

1. Is ZBTB2 the only candidates that activate HIF-1 under P53-deficiency? The authors performed a two-step screening, and from the first-round screening, they identified 4 candidates. However, whether these 4 genes or others were subjected to the second round of screening is not clear. From the description from Line7-9, the authors not stated clearly what genes were used for the second round of screening, and related data was not presented. This indicates the rationale to choose ZBTB2 as the final candidate for further investigation.

In the two-step screening experiment, we conducted luciferase assays to quantify HIF-1 activity, and confirmed that ZBTB2 is the only gene that activates HIF-1 in p53-deficient cells, but not in p53-proficient cells, among the 4 candidate genes, ZBTB2, UCHL1, IDH3α, and LY6E. We added the data to the revised manuscript (Fig EV2A-B). In addition, we revised the manuscript to clearly explain the two-step screening method (Page 4, lines 5-13).

2. From the data from Fig. 1A, it seems like the HIF-1 activity elevated, instead of decreased, in the p53 functional (p53+/+) HCT116 cell versus p53-null (p53-/-) under

hypoxia, which is not consistent with the hypothesis and initial data from U2OS (Fig. S1). How to explain this discrepancy? Besides, how about the HIF-1a protein level in these conditions?

When we focus on the expression levels of ZBTB2, all of our data look consistent; namely, there is a possibility that whether p53 suppresses HIF-1 activity depends on the basal expression levels of ZBTB2. We confirmed that in HeLa cells, in which endogenous ZBTB2 expression was not high, p53 suppressed HIF-1 activity only when cells were exogenously introduced with the ZBTB2 expression vector (Fig 1A). Likewise, in U2OS cells, in which endogenous ZBTB2 expression was extremely high, HIF-1 activity was suppressed when p53 activity was induced by treatment with an MDM2 inhibitor, Nutlin-3a (Fig EV1). We discussed this point in Discussion of our revised manuscript (Page 12, lines 11-18).

As for the HIF-1α protein levels, immunoblotting in Figure 4A of our revised manuscript confirmed that ZBTB2 did not influence HIF-1α levels in either p53-proficient or -deficient cells.

3. Authors performed lots of gain-of-function by overexpression of ZBTB2 to confirm its oncogenic function in the p53 null content both in vitro and in vivo. However, from the therapeutic perspective of view, whether ZBTB2 is a potential target in the context of p53-deficient is unclear. One will be interested to see the consequence of cell proliferation and tumorigenesis after ZBTB2 genetic depletion either in vitro or in vivo.

Following this important suggestion from Reviewer #1, we additionally carried out several kinds of both *in vitro* and *in vivo* experiments and deepened our understanding about whether we can obtain a therapeutic effect by ZBTB2 inhibition. We employed the RNAi technique and ZBTB2 [1-113] inhibitory polypeptide for this purpose to strengthen our POC evidence and directly evaluate the usefulness of the inhibitory polypeptide. We found that proliferation of a functional p53-defcient cancer cell line, HeLa, was significantly delayed by silencing ZBTB2 *in vitro* (Fig 2I). ZBTB2 silencing significantly suppressed the invasiveness of U2OS cells (Fig 2E). The ZBTB2 [1-113] polypeptide inhibited the proliferation of cancer cells only when the expression of endogenous ZBTB2 was not silenced *in vitro* (Fig 6H). A tumor growth delay assay *in vivo* demonstrated that the ZBTB2 [1-113] polypeptide significantly delayed growth of the xenografted tumor with the functional p53-defcient cells (Fig 6I). We added these data to our revised manuscript (Figs 2E, 2I, 6H, and 6I).

4. The authors claimed that the homodimerization of ZBTB2 via its N terminus region increases the transactivation activity of HIF-1α. However, the causal relationship between ZBTB2 homodimerization and transactivation activity of HIF-1α is obscure. In other words, the current data could not fully support the conclusion that the N-terminal of ZBTB2, which is essential for its dimerization, is exclusively critical for HIF-1a

transactivation activity.

As the data from Fig. 3F clearly showed that other domains including BTB/POZ, ZF2, and ZF3 are also important and caused identical TAD activity loss as the N-terminus region. How to explain this phenomenon? Does the other domain also responsible for the dimerization? Either providing more direct evidence to claim or tune down the conclusion would be helpful.

In order to strengthen our conclusion that the N-terminal of ZBTB2, which is essential for its dimerization, is critical for HIF-1α transactivation activity, we additionally conducted *in vitro* experiments. The luciferase assay enabling us to quantify HIF-1α transactivation activity confirmed that the ZBTB2 N-terminus-mimetic polypeptide, ZBTB2 [1-113], which competitively inhibited ZBTB2 homodimerization, significantly suppressed the HIF-1α transactivation activity only when the expression of endogenous ZBTB2 was not silenced *in vitro* (Fig. 6D). In addition, we confirmed in our original data that the ZBTB2 status did not influence HIF-1α expression levels (Fig 4A), and that the ZBTB2 4A mutant, which cannot form a homodimer, failed to upregulate HIF-1α transactivation activity (Fig 5G). Based on these results, we collectively concluded that the N-terminal of ZBTB2, which plays a critical role in homodimerization, is essential for the activation of HIF-1α transactivation activity. We added the additional data to our revised manuscript (Fig 6D).

In order to analyze the reason why not only the deletion mutant lacking "the N-terminus 23 a.a. region" but also that lacking "the BTB/POZ domain", "ZF2", or "ZF3" did not upregulate the transactivation activity of HIF-1α, we additionally carried out some experiments. First, the ChIP-qPCR experiment confirmed that wildtype ZBTB2 was recruited to the promoter regions of the STC1 gene, whose expression is under the control of the ZBTB2-HIF-1 axis, but the ZF2- or ZF3-deletion mutant of ZBTB2 was not (Fig 4K). Moreover, the split luciferase complementation assay to evaluate ZBTB2 homodimer formation demonstrated that ZBTB2 protein lacking ZF2 or ZF3 still had an ability to form a homodimer (Fig 4L). These results collectively indicate that ZF2 and ZF3 function in the recruitment of ZBTB2 to the promoter regions of its target genes, but not in ZBTB2 homodimerization. In addition, molecular dynamics simulations suggested that the L3-L23 region, A86-L89 region, and P106-L113 regions are all located on the same plane, whose normal line is the C2 axis of the ZBTB2 homodimer (Fig 5A). These three regions may be responsible for the stability of the ZBTB2 homodimer (Fig 5F). All of these findings explain why not only the deletion mutant lacking "the N-terminus 23 a.a. region" but also that of "the BTB/POZ domain (24-117 a.a.)", "ZF2", or "ZF3" failed to upregulate the transactivation activity of HIF-1α. Based on these data, we revised our manuscript (Page 8, lines 11-21; Page 9, line 16-Page 10, line 5).

Minor comments

1. p53 overexpression in Fig. 1C is suggested to confirm either by western blotting or

qRT-PCR.

We performed Western blotting to confirm the overexpression of exogenous p53 and added the data to our revised manuscript (Fig 1C).

2. The results from Page5, Line24-27, and Page6, Line1-5 does not support the conclusion that ZBTB2 activate HIF-1a, but more fit to the section "Homodimerization of ZBTB2 via its N terminus region increases the transactivation activity of HIF-1α"

Following the kind suggestion, we moved the text and corresponding figures to the suggested part. As a result, Supplementary Figures S6 and S7 became Expanded View Figs EV4G and EV4H, respectively, in our revised manuscript.

3. Please show representative images for the invasion assays from Fig. 2D and 2E.

We added representative images as Expanded View Figure EV3B-EV3D of our revised manuscript.

4. The label is missing for the tumor growth showed in Fig. 2H.

We have put labels on the figure, Fig 2J, in our revised manuscript.

5. In the patient survival analysis from Fig. 2J and K, what's the percentage of p53 deficiency/loss-of-function mutation in these patients? Does the tissue microarray or colorectal cancer cohort contain information of p53 state? If so, it may be helpful to stratify the patient according to p53 state and re-examine the Kaplan-Meier analysis in these patients.

Following this suggestion, we stained the clinical lung cancer samples with anti-ZBTB2 and anti-p53 antibodies, and analyzed the correlation between the ZBTB2 expression levels and prognosis of patients stratified by the p53 status. Kaplan-Meier analysis revealed that high ZBTB2 expression levels were correlated with poor disease-free survival of patients with mutant p53 tumors ($p = 0.0416$), but not with wildtype p53 tumors ($p = 0.0976$; Fig 3C). We added these data to the revised manuscript.

As for Kaplan-Meier analysis using the web-based free analytical tool (PrognoScan: http://dna00.bio.kyutech.ac.jp/PrognoScan/), it was impossible to analyze the correlation between the ZBTB2 expression levels and prognosis in patients stratified by the p53 status; therefore, we decided to delete the data from the revised version of our manuscript.

Reviewer #2:

Overall. Koyasu et al. provide evidence that ZBTB2 is linked to HIF1a activation. This is supported by clinical correlation. Figures 1, 2, and 3 are well designed and provide compelling evidence for ZBTB2 and HIF1 activation.

We would like to thank you for this comment supporting the publication of our article in *EMBO Rep*. We revised our manuscript to fully accommodate the reviewer's comments. We hope the manuscript will now be found suitable for publication.

Specific Comment 1-1. A minor concern in Fig.3b is in HCT116 with ZBTB2 under "normoxia" and hypoxia. It appears to be a significant induction compared to EV. The authors should consider conducting statistical analysis on this data.

We conducted statistical analysis under normoxia, too, and confirmed that ZBTB2 overexpression increased transactivation activity of HIF-1α in the experimental setting of the luciferase assay, as pointed out by Reviewer #2 here. But this result is quite reasonable, and we could understand why ZBTB2 induced luciferase bioluminescence in this luciferase assay for HIF-1α transactivation activity, but did not in another luciferase assay for HIF-1 activity, by considering the differences in the principles of these two assays.

In the luciferase assay for HIF-1α transactivation activity, the cells were transfected with plasmid expressing the Gal4 DNA binding domain (Gal4 DBD) fused to the HIF-1α transactivation domain (TAD: HIF-1α 531-826 a.a.). Because TAD was introduced with a P564A mutation to avoid oxygen-dependent degradation, the Gal4 DBD-HIF-1α TAD P564A fusion protein, which undergoes the regulation by ZBTB2, exists even under hypoxia (regardless of oxygen conditions). Therefore, our result that ZBTB2 overexpression increased luciferase bioluminescence in this assay means that ZBTB2 has activity to stimulate HIF-1α transactivation activity even under normoxia if the TAD domain exists.

On the other hand, in the latter luciferase assay for HIF-1 activity, endogenous HIF-1α, which is rapidly degraded under normoxia, is responsible for luciferase expression from the 5HRE-luc reporter gene. Therefore, the reporter never expresses bioluminescence under normoxia, even if ZBTB2 tries to stimulate HIF-1α transactivation activity.

Here, we would like to explain our opinion regarding why ZBTB2 slightly upregulated HIF-1α transactivation activity even in the presence of p53 in Fig 4B. It would be reasonable to consider that the valance between the expression levels of p53 and those of ZBTB2 determines whether p53 fully suppresses the ZBTB2-HIF-1 axis. We considered that this might be a reason why p53 failed to fully suppress the ZBTB2-HIF-1 axis in Fig 4B, where Gal4 DBD-HIF-1α TAD P564A was exogenously overexpressed. By conducting an additional luciferase assay for HIF-1α transactivation activity, we could get supporting evidence for our assumption; HIF-1α transactivation activity was completely

suppressed when HCT116p53-/- cells were introduced with a large amount of p53 expression vector. We added these data to our revised manuscript (Fig EV4B).

Specific Comment 1-2. The authors use less than 0.1% oxygen which is anoxic. The authors should either conduct the experiments under hypoxic 1% or label the text as anoxic.

We changed the labels "normoxia" and "hypoxia" to the "absolute oxygen concentration, such as "20%" and "< 0.1%", in our revised manuscript.

Specific Comment 2. The authors show ZBTB2 will dimerize with itself in overexpression experiments. However, it is unclear what the physiological condition would be to trigger the dimerization. The authors should consider determining if the dimerization is evident under hypoxia (1% oxygen). The dimerization may be specific to different conditions.

In order to analyze whether ZBTB2 forms a homodimer under physiological conditions, we additionally carried out *in vitro* experiments.

When HeLa cells, in which endogenous ZBTB2 expression is moderate, were introduced with the inhibitory polypeptide for the ZBTB2 homodimerization, ZBTB2[1-113], HIF-1α transactivation activity and HIF-1 activity were significantly suppressed (Fig 6D and E). In addition, we confirmed that such inhibitory effects were observed when endogenous expression of ZBTB2 was not silenced (Fig 6D and E), indicating that endogenous ZBTB2 forms a homodimer under physiological conditions.

Moreover, we performed the split luciferase complementation assay to evaluate ZBTB2 homodimerization under hypoxia (<0.1% oxygen) as well as normoxia (20% oxygen), and confirmed that the dimerization efficiency was not influenced by oxygen conditions (Fig 4J).

We added these data to our revised manuscript.

Specific Comment 3. The authors measure tumor growth by volume over time which is important. However, the final tumor weight, examination of necrosis of the tumors, vascularization (new blood vessel formation vs. existing blood vessels), circulating tumor cells, and metastasis would help provide a physiological pathway that ZBTB2 is affecting HIF1 can influence many of these pathophysiological conditions.

Because it was difficult for us to perform every experiment suggested here, we conducted an *in vivo* experiment and particularly analyzed the impact of ZBTB2 on distant tumor metastasis. This is because we had already confirmed in our original manuscript that, whereas ZBTB2 did not influence the expression of angiogenesis-related genes, ZBTB2 induced the expression of a series of metastasis-

related genes, such as matrix metalloproteases, and was actually involved in invasion of p53-deficient cancers (Figs 2A,B,D-F, and EV3A-D). An *in vivo* model of pulmonary metastasis demonstrated that ZBTB2 facilitated distant metastasis of HCT116 p53-/- cells, but not that of HCT116 p53+/+ cells. We added these data to our revised manuscript (Figs 2G and EV3E).

We recognize the necessity of further studies to approach the points made by Reviewer #2. Therefore, we additionally discussed them in our revised manuscript (Page 13, lines 8-12).

Specific Comment 4. **It is unclear if the statement that wild type p53 can inactivate HIF1 in the context of nutlin. There are several reports that Mdm2 is necessary for HIF1 activity, and Nutlin will block the Mdm2-HIF1a interaction. Thus, the authors did not address if Nutlin influenced the p53-Mdm2 or Mdm2-HIF1 interaction. Considering this is outside the paper's focus, the authors should consider removing this data or provide a more comprehensive review of the literature in the discussion outlining the current understanding of the HIF1-Mdm2 and p53-mdm2 pathways under hypoxic conditions.**

Following the advice, we reviewed the current understanding of the relationships among HIF-1, p53m, and MDM2, and discussed how our findings approach the missing link in our revised manuscript (Page 12, lines 9-18).

Reviewer #3:

Overall. In this manuscript the authors have tried to bridge the molecular players that are involved between HIF-1-dependent hypoxia signaling and p53-deficiency. They claim to identify ZBTB2, a zinc finger DNA binding protein to be a key mediator in that pathway. Though the study may be interesting, the reviewer's enthusiasm is dampened due to the moderate impact of the study, as well as the study's flawed study design (over dependence on over expression system).

In order to brush-up our manuscript by following the constructive and helpful comments from Reviewer #3, we performed a series of loss-of-function studies, etc. We hope that not only Reviewers #1 and #2 but also Reviewer #3 will find our revised manuscript suitable for publication in *EMBO Reports*.

Major criticisms:

1. The authors have not established a direct correlation between the p53 deficiency and induction of HIF1A activity. In fact, there are studies that suggest that HIF1A stabilizes p53 activity (An et al, Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha; Nature 1998; 392(6674):405-8). It is thus counter intuitive, but not impossible, that loss of p53 would induce higher HIF1A activity, but the authors need to show that a significant correlation exist between these two.

Thank you very much for the constructive comment. In the present study, we confirmed that p53 and HIF-1α did not influence expression levels of each other, at least in our experimental setting (Fig 4A), whereas the p53 status negatively affected the HIF-1 activity in ZBTB2-expressing cells and decreased the expression of ZBTB2-HIF-1-downstream genes. Based on these results and, moreover, following Reviewer #3's comment, we decided to additionally analyze the influence of the p53 status on HIF-1 "activity" in clinical lung cancer samples by staining the sections with antibodies against both p53 and the most representative HIF-1-downstream gene, CA9. Fisher's exact test demonstrated that the proportion of CA9-expressing tumors was significantly larger in patients with ZBTB2-high tumors compared with those with ZBTB2-low tumors when p53 was mutated (Fig 3B). Thanks to the comment from Reviewer #3, we could successfully confirm that the relationships among the p53 status, ZBTB2 expression levels, and HIF-1 activity we hypothesized are correct in a clinical setting. We added the new data to our revised manuscript.

2. The screening that identified ZBTB2, along with UCHL1, IDH3a and LY6E as the candidates that can significantly upregulated HIF-1 activity was carried out in HeLa cells, which itself do not have any p53 activity (Figure S2). Thus, it looks like that the authors wanted to put a thumb on the scale here (choosing to use a p53 deficient cell

line to identify the candidates) and then making a case that ZBTB2 links p53 deficiency to HIF1A activation. In other words, the screen is not agnostic to the p53 status. It is not a major flaw, but the authors should disclose it right away that they were validating their candidates in a p53 compromised cell lines. This makes the Figure 1 redundant, or at best, the Figure 1 can be added to the Figure S2, to make the case that ZBTB2 increases HIF1A activity in p53 deficient isogenic cell lines. Otherwise, the logic is circular in nature, and the reviewer does not see its significance to merit a main Figure in the manuscript.

Our insufficient explanation in the original manuscript might have caused the misunderstanding of Reviewer #3. As suggested by Reviewer #3, we firstly confirmed that not only ZBTB2 but also UCHL1, IDH3α, and LY6E upregulated HIF-1 activity in functional p53-deficient HeLa cells (These results were demonstrated in our original manuscript). After that, we employed HCT116 p53-/- cells as well as HCT116 p53+/+ cells in order to select a candidate gene which enhanced HIF-1 activity only in p53-deficient cells. As a result, the luciferase assay to evaluate HIF-1 activity demonstrated that only ZBTB2 among the 4 candidate genes exhibited the expected activity to facilitate HIF-1 activity particularly in p53-deficent cells, but not in p53-intact cells. In order to fully explain our two-step screening strategy, we added the data of the second screening experiment using p53-proficient and p53-deficient cells to our revised manuscript (Fig EV2B) and revised the main text so as to fully explain the aim and strategy of our screening experiments in detail (Page 4, lines 5-13).

3. A major deficiency in the manuscript is that the authors have not established whether ZBTB2-HIF-1 axis is a major driver of aggressive tumor invasion. The studies that have been presented are to make a case of for the said axis to contribute to modest (though significant) increase in tumor invasion/proliferation. But the impact of the study is dampened by the failure of the authors to establish that this axis is necessary and sufficient to maintain and progress tumorigenesis. So, though the study may illuminate that ZBTB2-HIF1A is modulated by dimerization of ZBTB2, the significance of the observation is academic at best. The study is further complicated by the fact that ZBTB2 is ectopically over expressed. It would have been more interesting if the authors could identify cell lines that have high endogenous ZBTB2 expression, with concomitant high HIF1A activity, and carried out the loss of function assays in those cell lines.

Following this advice from Reviewer #3, we employed HeLa and U2OS cells, which expressed certain levels of endogenous ZBTB2, and additionally performed several kinds of loss-of-function studies *in vitro* and *in vivo*. Our original and additional data confirmed that the silencing of endogenous ZBTB2 significantly suppressed HIF-1α transactivation activity (Fig 4C), HIF-1 activity (Fig 1B),

HIF-1-dependent expression of matrix metalloproteases (Fig 2B), invasiveness of p53-deficient cancer cells (Fig 2E), and proliferation of cancer cells (Fig 2I). Moreover, when we inhibited the homodimer formation of endogenous ZBTB2 by the ZBTB2 N-terminus-mimetic polypeptide, ZBTB2 [1-113], the inhibitory peptide significantly suppressed HIF-1α transactivation activity (Fig 6A), HIF-1-dependent expression of matrix metalloproteases (Fig 6C,F,G), proliferation of p53-deficient cancer cells (Fig 6H), and growth of p53-deficient xenografted tumors (Fig 6I). We demonstrated all of these data in our revised manuscript.

4. The Kaplan Meier analysis shown in 2J and 2K correlating the high expression of ZBTB2 with relatively poor outcome is over simplistic. To support their claim, the authors need to find out the p53 and the HIF1A status in the samples as well. Furthermore, the tumors in the TMA are expected to have significant tumor heterogeneity. It is interesting to note that the increased staining ZBTB2 in the tissue microarray are not necessarily in the invading front? Regardless, it should be doable to find out the p53 status of the cells staining strongly for the ZBTB2, as well as their HIF1A expression.

Following this suggestion, we performed immunohistochemical analysis for ZBTB2, p53, and one of most representative HIF-1-downstream genes, carbonic anhydrase 9 (CA9), in human lung cancers. Tumor samples were categorized into two groups, “ZBTB2 low” and “ZBTB2 high”, according to the expression levels of ZBTB2 in each wildtype p53 and mutant p53 patient (Fig 3A). Fisher’s exact test demonstrated that the proportion of CA9 expressing tumors was significantly larger in patients with ZBTB2-high tumors compared with those with ZBTB2-low tumors when p53 was mutated (Fig 3B).

We next analyzed the correlation between the ZBTB2 expression levels and prognosis in patients stratified by the p53 status (Fig 3C). Kaplan-Meier analysis revealed that high ZBTB2 expression levels were significantly correlated with poor disease-free survival of patients with mutant p53 tumors (p = 0.0416), but not with wildtype p53 tumors (p = 0.0976; Fig 3C). We added these data to our revised manuscript.

As for Kaplan-Meier analysis using the PrognoScan database (http://dna00.bio.kyutech.ac.jp/PrognoScan/) (Fig. 2K in our original manuscript), it was impossible to analyze the correlation between the ZBTB2 expression levels and prognosis in patients stratified by the p53 status because of the limitation of the free web analysis tool; and therefore, we decided to delete the data from the revised version of our manuscript.

5-1. The study's overall dependence on overexpression of ZBTB2 is a major problem. Ectopic over expression of ZBTB2 is most likely not representative of the physiological ZBTB2 levels in the cancer cells. Also, the study depends highly on the

assays derived from the HRE reporter systems.

5-2. Is there a reason that the authors did not opt for a more agnostic approach, for example, transcriptomics analysis?

Following this comment, we carried out loss-of-function studies to suppress endogenous ZBTB2 using the RNAi technique and ZBTB2 N-terminus-mimetic polypeptide, ZBTB2 [1-113], as described in our response to Reviewer #3's Comment 3. We demonstrated all of the data in our revised manuscript (Figs 1B, 2B, 2E, 2I, 4C, 6A, 6C, and 6F-6I). Moreover, we extensively conducted qRT-PCR experiments in order to validate our results obtained using the reporter systems.

We additionally performed TCGA analysis. When we detected p53 mutation as low expression levels of the representative p53 downstream genes, such as BAX, ZMAT3, and CEACAM1, overall survival of patients with both ZBTB2 high expression and p53 mutation in their tumor was found to be poor compared with patients with other types of tumors (Fig 3D-F, right), whereas the p53 status itself did not influence overall survival of patients (Fig 3D-F, left). We added these data to our revised manuscript.

6. The biochemical assays showing that the homodimerization of ZBTB2 is required for transactivation of HIF1A again relies extensively on the over expression systems. The reviewer opines that a cleaner system is required to derive the conclusions stated, either by engineering of the endogenous ZBTB2 loci with the stated modifications, or by using cell lines where the endogenous ZBTB2 is inactivated. Overall, the study design is problematic, since it overwhelmingly depends on an overexpression system.

In order to analyze whether the ZBTB2 homodimerization is required for the increases in transactivation activity of HIF-1α under physiological conditions, we additionally carried out *in vitro* experiments, as we explained in our response to Reviewer #2's Specific Comment 2.

When HeLa cells, in which endogenous ZBTB2 expression is moderate, were introduced with the inhibitory polypeptide for ZBTB2 homodimerization, ZBTB2[1-113], HIF-1α transactivation activity and HIF-1 activity were significantly suppressed (Fig 6D and E). In addition, we confirmed that such inhibitory effects were observed when endogenous expression of ZBTB2 was not silenced (Fig 6D and E), indicating that homodimerization of endogenous ZBTB2 plays an important role in the activation of HIF-1. We added these data to our revised manuscript.

1st Revision - Editorial Decision **4th Oct 2022**

Dear Prof. Harada,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that I asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of your study.

Before proceeding with formal acceptance, I have these editorial requests I ask you to address in a final revised manuscript:

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section. Please also remove the conflict of interests statement from the title page.

- We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. Thus, please remove the author contributions section from the manuscript text file. See also guide to authors:
https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

- I would suggest uploading the present Dataset EV1 (with the cloning primers) as table. Please name this Table EV1 and add a callout for this to the manuscript text file. Finally, please add a legend for the table (with a title) at the end of the manuscript (see below).

- Please order the manuscript sections like this (using these names):
Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials & Methods - DAS - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends - Expanded View Table legend

- Please upload also the EV figures in production quality (best resolution possible) as separate, individual files (in .tif format - like the main figures) upon re-submission.

- Please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images themselves. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently, no scale bars have been provided (see e.g. Figs. 3A and EV3).

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main, EV and Appendix figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams (main, EV and Appendix figures). Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics.

- The two source data files (atomic coordinates) are datasets. Please name these Dataset EV1 and Dataset EV2, provide these as excel files and add legend on the first TAB of the excel file. Finally, please add callouts for these to the manuscript text file.
- Thank you for providing the source data. Please upload this as single files, one pdf file per figure (main and EV figures).

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

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short bullet points highlighting the key findings of your study (two lines each).
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

This revision has been obviously strengthened by adding new loss-of-function studies from both in vitro and in vivo, other comments from the reviewer were also properly addressed. I have no further comments and think it is suitable for publication.

Referee #2:

The authors have addressed my concerns.

Referee #3:

The authors have adequately addressed the previous critiques, and the manuscript should be considered for publication.

The authors have addressed all minor editorial requests.

Prof. Hiroshi Harada
Kyoto University
Graduate School of Biostudies
Yoshida Konoe-cho
Sakyo-ku
Kyoto 606-8501
Japan

Dear Prof. Harada,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: 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." Please note that the author checklist will still be published even if you opt out of the transparent process.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

****

THINGS TO DO NOW:

Please note that you will be contacted by Wiley Author Services to complete licensing and payment information. The required 'Page Charges Authorization Form' is available here: https://www.embopress.org/pb-assets/embo-site/er_apc.pdf - please download and complete the form and return to embopressproduction@wiley.com

EMBO Press participates in many Publish and Read agreements that allow authors to publish Open Access with reduced/no publication charges. Check your eligibility: https://authorservices.wiley.com/author-resources/Journal-Authors/open-access/affiliation-policies-payments/index.html

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2021-54042V3 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

EMBO Press Author Checklist

Corresponding Author Name: Hiroshi HARADA, Ph.D.	
Journal Submitted to: EMBO Reports	
Manuscript Number: EMBOR-2021-54042	

USEFUL LINKS FOR COMPLETING THIS FORM
The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January 2022)
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data
The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	No restriction

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

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

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

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	provided every information
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Yes	provided every information
Please detail **housing and husbandry conditions**.	Yes	provided every information

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

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Yes	provided every information

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

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

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

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

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Reproducibility of results was confirmed by repeating the same experiments at least three times. We described it in the main text (Materials and Methods).
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	well described

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Yes	described in the main text
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Yes	described in the main text
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	described in the main text
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Yes	described in the main text

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

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

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

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