

Combination of AID2 and BromoTag expands the utility of degron-based protein knockdowns

Yuki Hatoyama, Moutushi Islam, Adam Bond, Ken-ichiro Hayashi, Alessio Ciulli, and Masato Kanemaki

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Dear Prof. Kanemaki,

Thank you for the submission of your manuscript to EMBO reports. So far, I could only secure 2 referees for it and we have now received the enclosed reports from both of them. Given that they are in fair agreement, I am making a decision on your ms now based on the 2 reports we have and in the interest of time.

As you will see, both referees acknowledge that the findings are interesting. Both only have a few more suggestions for how the manuscript could be further improved and strengthened and I think all suggestions are good and should be addressed. Please let me know if you disagree or have any questions or comments. We can also discuss the exact revision requirements in a video chat, if you like.

I also paste below some comments from an advisor I contacted before sending your ms for peer-review. Please also consider this comment in your revised ms text.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns 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 major 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 realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (13th Jul 2024). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

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- 2) Your manuscript contains statistics and error bars based on $n=2$. Please use scatter blots in these cases. No statistics should be calculated if $n=2$.

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.

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2) individual production quality figure files as .eps, .tif, .jpg (one file per figure). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

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- 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: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

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4) 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.

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6) 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

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7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see <https://www.embopress.org/page/journal/14693178/authorguide#datadeposition>). 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 (see also <https://www.embopress.org/page/journal/14693178/authorguide#datadeposition>). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *

If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

9) Our journal also 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 <https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>

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- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.),
- If the data are obtained from n Program fragment delivered error ``Can't locate object method "less" via package "than" (perhaps you forgot to load "than"?) at //ejpvfs23/sites23b/embor_www/letters/embor_decision_revise_and_review.txt line 56.' 2, use scatter blots showing the individual data points.

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 also include scale bars in all microscopy images.

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

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I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely,

Referee #1:

In this manuscript, Hatoyama et al use 3 different degron tagging systems, either individually to degrade a reporter, or in combination to degrade proteins involved in higher order chromosome structure or DNA replication. They show that

- Individual degron tags sometimes fail to degrade proteins known to be essential to a sufficient degree to reveal the null phenotype
- Different degron tags, and different ligands for the same degron tag, can degrade a reporter protein with different efficiencies, and with profoundly different efficiencies of recovery
- Different degron tags can be used to exert orthogonal control over two distinct proteins
- Different degron tags fused to the same protein can act synergistically to induce more complete degradation, revealing null phenotypes
- Tandem or triple mAID tags greatly reduce the steady-state expression of fusion proteins in a Tir1-independent manner
- Cells simultaneously depleted of ORC1 and CDC6 enter mitosis without undergoing DNA replication.

The experiments are well performed and the conclusions, although in some cases not entirely novel or surprising, are justified. The authors should address the following comments before publication.

- 1) The slow recovery following dTAG- and BromoTAG-mediated depletion is a potentially significant finding of importance to understanding PROTAC pharmacology as well as informing researchers on which degron tag system to use. This section therefore deserves to be developed further. The authors should repeat the recovery experiment using the dTAG-13 ligand, for which efficient reversibility has been demonstrated by others (e.g. Nabet et al Nature Chem Bio 2018, Abuhashem et al Dev Cell 2022). Longer exposure times could be used to drive more complete dTAG-13-mediated degradation than shown in Figure 2.
- 2) The authors explanation for poor recovery is that the PROTAC ligands may remain associated with their substrate receptors to provide a washout-resistant ligand pool. If true, this pool might be reduced and reversibility might be more efficient if degradation is first achieved using lower ligand doses, over longer exposure times if necessary. This should be tested - can recovery be improved?
- 3) In Figure 6, it is stated that the double and triple mAID tag destabilises the target protein, but only steady-state expression data are presented. Can the authors show via proteasome inhibition or similar that the 2mAID and 3mAID-ORC1 fusions are less stable than the single tag?
- 4) Work demonstrating a very similar concept - combining AID with another degron tag in order to reveal null phenotypes via more complete degradation - was published some time ago by the Hochegger and Lindqvist groups (PMID: 32350921, PMID: 30008317, PMID: 34972955), including one of the same target proteins (CDC6) as the current study. Although one of these papers is cited (line 329), the authors should more explicitly acknowledge this prior work on double degrons.
- 5) Line 80 "Because IMiDs inevitably induce off-target proteolysis of CRBN neo-substrates". This statement may need to be updated in light of the recent Science paper from David Liu's group

Referee #2:

This is a timely and interesting study by Hatoyama et al. that covers two important aspects that are of interest to the wider cell biology community. Firstly the paper gives a detailed quantitative comparison of the most up-to-date induced deflation systems and sets a precedent for the use of double mAID-Bromo degron tags. Secondly, the study addresses an important question in the DNA replication field: Is Orc1 an essential replication licensing protein? Using the double degron method the authors show that only enhanced depletion of Orc1 causes a complete block in proliferation but even in these circumstances origins still manage to fire albeit in an erroneous manner.

Both points are important and I am confident that this MS will be well received by many researchers interested in the degron technology, as well as by the DNA replication field.

The study is technically sound and the experiments are mostly well performed and documented.

I only have a few minor comments and one suggestion for an additional experiment. Other than that I would support the publication of this study in EMBO reports

Minor comments:

1) The Lemmens et al. study that is cited in the discussion sets an actual precedent for this study both in terms of findings on Cdc6 requirements for DNA replication, uncoupling of S and M-phase and double degron tags (in this case Smash-AID) enhancing degradation. I think it would be fair to discuss the Lemmens paper in the introduction in the context of this study rather than bury this paper in a brief comment in the discussion.

2) A technical comment: FACS data and colony assays should be quantified from data from at least 3 repeats. For example, in Figure 5E, what is the proportion of S-phase cells? How reproducible is this across three biological repeats? Likewise, median points of three independent experiments should be shown in the MSM loading assays (E.g Figure 5F) and p-values should be calculated on the three median points rather than the large number of single-cell data that generate very small p-values for small differences in the data.

3) Suggested experiment: I'm still puzzled by the fact that even the strongest possible depletion of Orc1 does not abolish DNA replication. Are a few molecules of Orc1 enough to support licensing, or is there a true parallel mechanism involving Cdc6 and Orc1? This will be hard to determine, but the authors should try and further enhance Orc1 depletion by combining mRNA degradation using siRNA and depletion of the already synthesised protein with the double degron. This will reduce synthesis rates as well as enhance degradation and ultimately cause maximum depletion of Orc1. Will this be sufficient to cause a complete block in replication?

Advisor's comment:

The effect of co-depleting ORC1 and CDC6 is interesting to me as a member of the replication field, as the data are so clear, yet the interpretation is complicated by the fact that the phenotype comes from depleting two proteins, so one can't conclude whether ORC1 is absolutely essential for MCM loading (it almost certainly is) and the challenge is just to deplete it sufficiently well, or whether the key thing is to deplete both ORC1 and CDC6 (e.g. if other pathways for MCM loading were to exist, though until now no such pathway has been identified).

Our response to Reviewers and Advisor

(The comments are shown in blue, while our responses are shown in black.)

Response to Reviewer 1

We sincerely thank this reviewer for giving constructive comments and raising the issues to improve the manuscript.

1) The slow recovery following dTAG- and BromoTAG-mediated depletion is a potentially significant finding of importance to understanding PROTAC pharmacology as well as informing researchers on which degron tag system to use. This section therefore deserves to be developed further. The authors should repeat the recovery experiment using the dTAG-13 ligand, for which efficient reversibility has been demonstrated by others (e.g. Nabet et al Nature Chem Bio 2018, Abuhashem et al Dev Cell 2022). Longer exposure times could be used to drive more complete dTAG-13-mediated degradation than shown in Figure 2.

Following your comments, we tested whether more prolonged exposure to dTAG-13 would drive more depletion. This was indeed the case (Figure S2C). We also carried out the recovery experiment with 100 nM dTAG-13 after 12 h exposure and found that the recovery was not good (see Figure for Reviewer 1, panel A). We did not include this data because we tested recovery data with a lower concentration of 5-Ph-IAA, dTAGv-1 and AGB1, as you will see in our following response.

2) The authors explanation for poor recovery is that the PROTAC ligands may remain associated with their substrate receptors to provide a washout-resistant ligand pool. If true, this pool might be reduced and reversibility might be more efficient if degradation is first achieved using lower ligand doses, over longer exposure times if necessary. This should be tested - can recovery be improved?

We carried out experiments and added new data (Figure S3B). In these experiments, we treated the reporter cells with the DC50 concentration of each ligand identified in Figure 2B. After 12 h treatment, the GFP expression level became approximately 30%, and we started the recovery from this time point. As you predicted, the recovery became better than shown in Figure 2D. However, the overall trend was the same: The recovery with AID2 was better than the other two.

3) In Figure 6, it is stated that the double and triple mAID tag destabilises the target protein, but only steady-state expression data are presented. Can the authors show via proteasome inhibition or similar that the 2mAID and 3mAID-ORC1 fusions are less stable than the single tag?

Thanks for pointing it out. We also wanted to understand the reason why 2mAID and 3mAID lowered the expression level of ORC1. So, we investigated the half-life of the fusion protein by adding cycloheximide and the mRNA level by qPCR (Figure S7C, D). We found that 3mAID-ORC1 has a shorter half-life compared to mAID- and 2mAID-ORC1 (Figure S7C). We are surprised to see that the mRNA level of 2mAID-ORC1 was significantly reduced (Figure S7D), suggesting the insertion of 2mAID to the N-terminal coding region of the *ORC1* gene affected transcription or mRNA

stability. Both 2mAID and 3mAID reduced the expression of ORC1, but the mechanism might differ. We wrote these findings in the main text (lines 379-383 on page 16).

4) Work demonstrating a very similar concept - combining AID with another degron tag in order to reveal null phenotypes via more complete degradation - was published some time ago by the Hochegger and Lindqvist groups (PMID: 32350921, PMID: 30008317, PMID: 34972955), including one of the same target proteins (CDC6) as the current study. Although one of these papers is cited (line 329), the authors should more explicitly acknowledge this prior work on double degrons.

We agree that we should have acknowledged their prior work on AID-SMASH. So, we changed Introduction accordingly and added citations (lines 104-106 on page 5).

One thing that we noted was that the AID-SMASH system did not work for CDC6 in HCT116 cells, even though we used the same tagging construct that came from Dr. Lindqvist (Figure for Reviewer 1, panels B and C). It seemed that a cleaved CDC6 proteins without the mAID-SMASH tag was produced (shown by red arrow, panel B) in the cells, and this protein was not degraded and supported cell growth (panel C). We wanted to compare our AID2-BromoTag with AID-SMASH, but we could not do it.

5) Line 80 "Because IMiDs inevitably induce off-target proteolysis of CRBN neo-substrates". This statement may need to be updated in light of the recent Science paper from David Liu's group

Thanks for letting us know. We changed the text accordingly in Introduction and cited the paper (lines 81-83 on page 4).

Response to Reviewer 2

We are delighted to read your comments stating, "I am confident that this MS will be well received by many researchers interested in the degron technology, as well as by the DNA replication field". We appreciate you taking the time to evaluate our manuscript and provide us with constructive comments and questions.

1) The Lemmens et al. study that is cited in the discussion sets an actual precedent for this study both in terms of findings on Cdc6 requirements for DNA replication, uncoupling of S and M-phase and double degron tags (in this case Smash-AID) enhancing degradation. I think it would be fair to discuss the Lemmens paper in the introduction in the context of this study rather than bury this paper in a brief comment in the discussion.

Thanks for pointing it out. Please read our response to point 4 from Reviewer 1.

2) A technical comment: FACS data and colony assays should be quantified from data from at least 3 repeats. For example, in Figure 5E, what is the proportion of S-phase cells? How reproducible is this across three biological repeats? Likewise, median points of three independent experiments should be shown in the MSM loading assays (E.g Figure 5F) and p-

values should be calculated on the three median points rather than the large number of single-cell data that generate very small p-values for small differences in the data.

We changed the figures showing the loading level of MCM and added statistical information. Please look at Figures 5F, 6D, S6F and S7E.

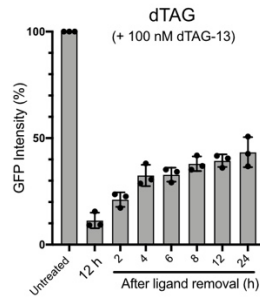
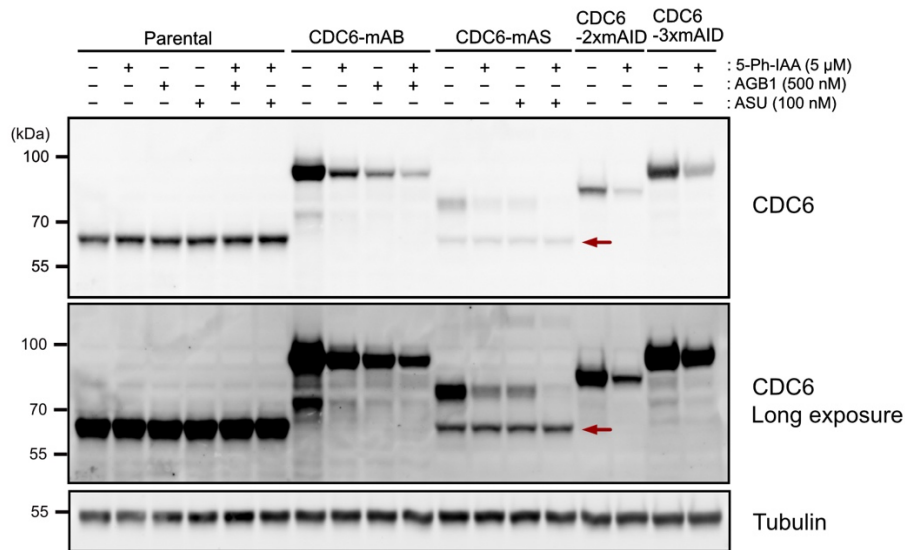
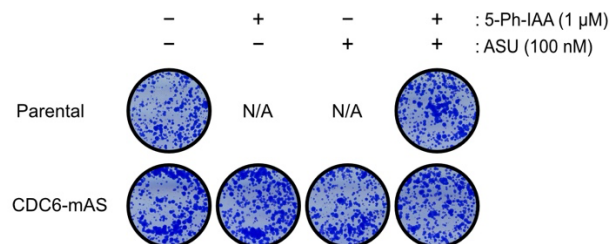
3) Suggested experiment: I'm still puzzled by the fact that even the strongest possible depletion of Orc1 does not abolish DNA replication. Are a few molecules of Orc1 enough to support licensing, or is there a true parallel mechanism involving Cdc6 and Orc1? This will be hard to determine, but the authors should try and further enhance Orc1 depletion by combining mRNA degradation using siRNA and depletion of the already synthesised protein with the double degron. This will reduce synthesis rates as well as enhance degradation and ultimately cause maximum depletion of Orc1. Will this be sufficient to cause a complete block in replication?

We thought that the raised issue was important. So, we decided to conduct the suggested experiments (Figure S8A). By combining AID2-BromoTag with siRNA, we depleted ORC1 and CDC6 to near the detection limit by WB (Figure S8B). In both cases, the combination of siRNA and AID2-BromoTag achieved more pronounced defects in S phase progression (Figure S8C, D), showing that MCM loading still occurred when ORC1 or CDC6 was depleted only by AID2-BromoTag (Figures 5E and S6E). Intriguingly, the combinational depletion of CDC6 induced almost complete suppression of DNA replication (Figure S8D), while that of ORC1 still allowed some DNA replication (Figure S8C). These data suggest two possibilities, which are hard to clarify: there was still ORC1 existing even after the combinational depletion, or MCM-loading occurred to some extent in the absence of ORC1. However, please note that ORC1 is pivotal to achieving MCM loading enough to drive cell proliferation. In this regard, ORC1 is essential for cell viability. In contrast, CDC6 is likely absolutely essential for MCM-loading. We added texts in Results and Discussion accordingly (lines 412-426 on page 17 and 537-540 on page 22).

Response to Advisor's comments

The effect of co-depleting ORC1 and CDC6 is interesting to me as a member of the replication field, as the data are so clear, yet the interpretation is complicated by the fact that the phenotype comes from depleting two proteins, so one can't conclude whether ORC1 is absolutely essential for MCM loading (it almost certainly is) and the challenge is just to deplete it sufficiently well, or whether the key thing is to deplete both ORC1 and CDC6 (e.g. if other pathways for MCM loading were to exist, though until now no such pathway has been identified).

Thanks for pointing out this important issue. Please read our comments to point 3 from Reviewer 2 and take a look at Figure S8.

A**B****C****Figure for Reviewer 1**

(A) The HCT116 reporter cells were treated with 100 nM dTAG-13 for 12 h. Subsequently, the cells were washed and the recovery of GFP was monitored up to 24 h. (B) Comparison of CDC6-mAID-SMASH with the other double-degrons. We generated HCT116 CDC6 degron cell lines by fusing mAID-BromoTag (mAB), mAID-SMASH (mAS), 2mAID or 3mAID to the C-terminus of CDC6. CDC6 levels in cells treated with the indicated ligands. In the CDC6-mAID-SMASH cells, a smaller CDC6 protein shown by red arrow was not degraded. Because we confirmed that the both CDC6 alleles are tagged with mAID-SMASH, it is likely that the mAID-SMASH tag was cleaved after translation. (C) The CDC6-mAID-SMASH cells did not show growth defects in when treated with the inducing ligands.

Dear Prof. Kanemaki

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees and I am happy to say that both support its publication now.

Only a few more minor editorial requests will need to be addressed before we can proceed with the official acceptance of your manuscript:

- Please upload the final ms file without any figures.
- Please correct the conflict of interest subheading to "Disclosure and Competing Interests Statement"
- We received an automatic note for Adam Bond about no longer being at ICR. Please send us his current email with the final ms file.
- We need from you a completed author checklist, which you can download from our author guidelines <<https://www.embopress.org/page/journal/14693178/authorguide>>. The completed author checklist will also be part of your transparent peer-review process file.
- The funding info on Young Scientists (DC2) and MEXT scholarship are missing in our online ms submission system, please add it with the final submission.
- Please upload the 3 tables as EV tables (Table EV1, etc) and correct their names in the table files and in the manuscript callouts.
- The APPENDIX FILE needs a table of content with page numbers; and the nomenclature (and the ms callouts) needs to be corrected to Appendix Figure S1, etc.
- The source data need to be uploaded as one (zipped) folder per figure and can have single files per figure panel.
- Materials & Methods should be called just Methods.
- During our figure check of accepted ms, we found a possible reuse within Figure 5B / Tubulin (left side): 5-Ph-IAA & AGB1. The blots are very similar in the figure. Blots are different in the source data than in the figure. Can you please check your figure/source data and explain what happened and redraw the figure if necessary.
- Please add the exact p values in the legends of figures 5f; 6d.
- Although 'n' is provided, please describe the nature of entity for 'n' in the legends of figures 2b; 3d; 4a-b.

I made a few minor changes to the abstract that needs to be written in present tense. Please let me know whether you agree with the following:

Acute protein knockdown is a powerful approach to dissecting protein function in dynamic cellular processes. We previously reported an improved auxin-inducible degron system, AID2, but recently noted that its ability to induce degradation of some essential replication factors, such as ORC1 and CDC6, was not enough to induce lethality. Here, we present combinational degron technologies to control two proteins and enhance target depletion. For this purpose, we initially compare PROTAC-based degrons, dTAG and BromoTag, with AID2 to reveal their key features and then demonstrate control of cohesin and condensin with AID2 and BromoTag, respectively. We develop a double-degron system with AID2 and BromoTag to enhance target depletion and accelerate depletion kinetics and demonstrate that both ORC1 and CDC6 are pivotal for MCM loading. Finally, we show that co-depletion of ORC1 and CDC6 by the double-degron system completely suppresses DNA replication, and the cells enter mitosis with single-chromatid chromosomes, indicating that DNA replication is uncoupled from cell cycle control. Our combinational degron technologies will expand the application scope for functional analyses.

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I look forward to seeing a final version of your manuscript as soon as possible.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors have added data and text to their revised manuscript which improves the quality, and addresses all of my concerns. Congratulations on a nice piece of work.

Referee #2:

The author has addressed my concerns and produced interesting new results. The paper should now be published and will be an important contribution to cell biology.

The authors have addressed all minor editorial requests.

Prof. Masato Kanemaki
National Institute of Genetics
Department of Chromosome Science
Yata 1111
Mishima 411-8540
Japan

Dear Prof. Kanemaki,

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.

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Yours sincerely,

Esther Schnapp, PhD
Senior Editor
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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
- 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

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 χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

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	Materials and Table EV1
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	Materials and Table EV3
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	Materials
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	Material and Table EV2
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	Table EV2
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.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail housing and husbandry conditions .	Not Applicable	
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.	Not Applicable	
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.	Yes	Methods, Saito & Kanemaki Current Protocols (https://doi.org/10.1002/cpz1.219)

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.	Yes	Figure legends, Methods
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?	Not Applicable	
Include a statement about blinding even if no blinding was done.	Not Applicable	
Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not Applicable	
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	Figure legends, Methods

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	Figure legends
In the figure legends: define whether data describe technical or biological replicates .	Yes	Figure legends

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).	Not Applicable	
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.	Not Applicable	
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).	Not Applicable	
Studies involving specimen and field samples : State if relevant permits obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

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?	Yes	Source data
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	