EMBO reports

The RNA polymerase II subunit Rpb9 activates ATG1 transcription and autophagy

Ting Huang, Gaoyue Jiang, Yanbing Zhang, Yuqing Lei, Shiyan Liu, Huihui Li, and Kefeng Lu
DOI: 10.15252/embr.202254993

Corresponding author(s): Kefeng Lu (lukf@scu.edu.cn)

Review Timeline:

Submission Date:	7th Mar 22
Editorial Decision:	21st Apr 22
Revision Received:	18th Jul 22
Editorial Decision:	9th Aug 22
Revision Received:	23rd Aug 22
Accepted:	26th Aug 22

Editor: Esther Schnapp/Martina Rembold

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.)

Dear Dr. Lu

Thank you for the submission of your research manuscript to our journal. Since my colleague Esther Schnapp is currently traveling, I have temporarily taken over the handling of your manuscript. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also point out several technical concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed.
Please note that we also ask from the editorial side to include all data that is relevant to the study, i.e., the screening results should be included and described in more detail.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and 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 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 (July 21, 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

**** 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. If you have not deposited any data, please add a sentence to the data availability section that explains that.

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.

When submitting your revised manuscript, we will require:

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).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

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.

4) a complete author checklist, which you can download from our author guidelines (). 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 (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

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:

- 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) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) Figure legends and data quantification:

The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- 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 {less than or equal to} 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.

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please also include scale bars in all microscopy images.

9) 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 .

10) 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 .

11) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. 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.

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."

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 form of your manuscript when it is ready. Please use this link to submit your revision:
https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Martina Rembold, PhD

Senior Editor
EMBO reports

****

Referee #1:

In this manuscript, Huang et al. identified the RNA polymerase II subunit Rpb9 as a transcriptional activator responsible for the upregulation of the autophagy-initiating kinase Atg1 in S. cerevisiae. Disruption of Rpb9 in yeast resulted in deficiency of autophagy, whereas rpb9Δ cells did not show any defects in endocytosis pathways. Rpb9 is a subunit of the RNA polymerase II, but rpb9Δ cells showed no significant effect on global transcription, rather only ATG1 transcription is specifically affected. Consistent with this, exogenous expression of HA-Atg1 could rescue autophagic defect in rpb9Δ cells. Rpb9 is thought to bind to the promoter region of the ATG1 gene together with Gcn4. Importantly, Rpb9 is evolutionarily conserved and these Rpb9 orthologs could rescue autophagic defect in rpb9Δ yeast cells. The findings of this study are conceptually important and shed new light on the transcriptional activation of autophagy-related genes. However, this reviewer thought that the data represented in this paper are somewhat preliminarily and there are several major issues to answer.

Major comments:
(1) In Figure 2, the authors did not determine which processes of autophagy are inhibited by disruption of Rpb9. Which processes are inhibited, Atg13 dephosphorylation, the PAS formation, Atg1 autophosphorylation, Atg9 recruitment, membrane elongation, or fusion with vacuoles? In addition, exogenous expression of HA-Rpb9 should be examined in these suggested experiments.

(2) Related to Figures 1G and 3D, protein expression levels of Atg1 (and other Atg proteins such as Atg13 and Atg17) should be investigated, because there is no data showing the relationship between the transcription level and translation level of Atg1 in WT and rpb9Δ cells.

(3) In Figure 2A, both experimental conditions and results of the high-throughput screen are not shown. This reviewer wondered whether all of 4857 non-essential genes were examined or only transcriptional compontns were examined?

(4) In Figure 2B etc., the authors used GFP-50Q as an autophagy reporter, however, the GFP-50Q reporter may be a "selective" substrate. If GFP-50Q is a selective autophagy reporter, the defects observed in rpb9Δ cells also indicate the defects in selective autophagy, not normal autophagy. GFP-Atg8 or Pgk1-GFP should be used to investigate normal autophagic activity. Alternatively, appropriate references showing that GFP-50Q is a normal autophagy reporter should be cited. (Related to the minor comment (3), this reviewer could not know the detail of the 50Q construct.)

(5) In Figure 5, EMSA using both Rpb9 and Gcn4 should be examined, because there is no direct evidence indicating the Rpb9 binding to the promoter region of the ATG1 gene.

(6) In Figure 6G, autophagic activities should be examined in POLR2I knockdown mammalian cells. The Rpb9 orthologs and transcriptional regulation of the ATG1/ULK1/3 gene were conserved, but its importance was not elucidated.

(7) In Figure 1F, please explain how to validate the results of the mRNA expression sequencing.

Minor comments:

(1) In Figure 1D, it is difficult to understand the experimental conditions. Does "R -> T" mean "R (3 h, 2nd lane) -> R+T (13 h, 3rd lane)"? If so, why the GFP processing efficiencies are so different between Figure 1D (2nd lane) and Figure 1B (2nd lane). If "R (3 h, not shown) -> R+T (3 h, 2nd lane; 16 h, 3rd lane)" is correct, the "R (3 h)" data should be shown. Experimental conditions should be described in detail.

(2) In Figure 2D, this reviewer could not understand why vam3Δ and ypt7Δ were used. What do the authors mean by these results? Rather, exogenous expression of HA-Rpb9 should be included in Figure 2D, 2E, 2F, and 3D as well as Figure 2B.

(3) This reviewer could not find the constructs of the HA-Atg1 expression plasmid, etc. and the 50Q construct. What vector was used and what promoter was used?

(4) In Figure 2H, why the GFP moieties are not detected?

(5) In Figure legend 2, (D) in line 626 is (E). The same applies thereafter.

Referee #2:

In this study, Huang T et al examined the mechanisms in control of ATG1 transcription in yeast via high-throughput screen in yeast. They discovered a key role of an RNA polymerase II subunit Rpb9 in this process and similar mechanisms were also found in mammalian cells. At present, most of the studies on ATG are on their post-translational modifications (PTMs) and thus this study reveals a new window in study of autophagy mechanisms.

Major comments:
1. Regarding the inhibitory effect of CHX on autophagy, the authors need to be more cautious on their conclusions, based on the fact that CHX has indirect effect on autophagy by increasing the intracellular AA concentration and subsequent activation of mTORC1. Therefore, the authors need to use additional method with additional data to support the conclusion that "autophagy induction depends on the synthesis of new proteins".
2. Figure 1: Authors also need to show the changes of Atg1 protein level, in corresponding to the changes of Atg1 mRNA level.
3. Figure 3C: In addition to Atg1, several other Atgs such as Atg5 and Atg13 were also down-regulated in rpb9 KO cells. On the other hand, the authors found that only Atg1 overexpression, but not Atg9, Atg13, and Atg17, can restore the defective autophagy in rpb9 KO cells. Here the authors need to address the following points: (i) Is rpb9 also implicated in the transcriptional regulation of Atg5 and Atg13? (ii) can the authors show the protein level of various Atg protein in the over-expression experiments? And (iii) can the authors test the effect of Atg5 which is also down-regulated?
4. Figure 5F: By examining the data carefully, actually over-expression of Gcn4 can partly restore autophagy in rpb9 KO cells, evidenced by the weak but obvious GFP band. In contrast, over-expression of rpb9 in Gcn4 KO cells failed to have the same effect, (Figure 5G). In both the text and figure legend, the authors failed to describe this difference.
5. Figure 5H: The model shown in this panel does not really reflect the data presented in the study, based on the comments in Point #4 above.

Referee #3:

Summary

In the manuscript by Huang et al. the authors demonstrate that the RNA polymerase II subunit Rpb9 regulates bulk autophagy in budding yeast by upregulating ATG1 transcription through binding of the transcription factor Gcn4. They furthermore show that the Rpb9 ortholog POLR2I also regulates ULK1 transcription in mammalian cells suggesting a conserved function for Rpb9.

Major Points

The authors should compare the Atg1 protein levels in nutrient-rich and nitrogen starvation medium (e.g. 3, 6 and 16 hours) for wild type and Rpb9 deleted yeast to show how changes in mRNA levels translate into changes in protein expression levels?

The authors should also study selective autophagy under nutrient-rich conditions (e.g. the Cvt pathway) in wild type and Rpb9 deleted cells.

To validate their model (Figure 5H) the authors should repeat the pulldown shown in Figure 5E with recombinant Rpb9 to demonstrate a direct interaction.

Minor Points

Figure 2H
The CPY1-GFP cleavage assay lacks a negative control. Can the author please include a strain with defective CPY processing?

Figure 2E
Ape1 should also be transported to the vacuole under nutrient-rich conditions. Hence, why can Ape1 not be found in the vacuole under those conditions?
The authors should repeat the experiments using a strain with untagged Ape1 and monitor Ape1 processing by Western blotting.

General

With the Atg1 protein expression levels in mind can the authors speculate in the discussion why the Atg1 expression levels in cells grown in nutrient-rich medium are sufficient to promote selective autophagy pathways such as the Cvt pathway but not bulk autophagy?

Can the authors show where the first zinc finger domain and the linker region in Rpb9 responsible for its discussed function are located with respect to the RNA polymerase?

In yeast as opposed to mammalian cells there is no bulk autophagy under nutrient-rich conditions. The authors should adjust the introduction accordingly.

Point-by-point responses to the referees

All the authors sincerely thank the referees for their time and effort. We are very pleased with the positive comments and helpful suggestions put forth by the referees, which have greatly improved the quality of this work. We have revised the manuscript accordingly and addressed all issues raised by the referees, as shown, point-by-point, below.

Referee #1

We thank the referee for the positive comments "***The findings of this study are conceptually important and shed new light on the transcriptional activation of autophagy-related genes.***". The specific and helpful comments are addressed as shown below.

Major comments:

1) "*(1) In Figure 2, the authors did not determine which processes of autophagy are inhibited by disruption of Rpb9. Which processes are inhibited, Atg13 dephosphorylation, the PAS formation, Atg1 autophosphorylation, Atg9 recruitment, membrane elongation, or fusion with vacuoles? In addition, exogenous expression of HA-Rpb9 should be examined in these suggested experiments.*"

Response: We thank the referee for this suggestion. As suggested, we observed the different processes of autophagy in Rpb9-deleted cells and in Rpb9-deleted cells with exogenous expression of HA-Rpb9. We examined PAS formation by observing the puncta of chromosomally GFP-tagged Atg1 or Atg13. The results showed that PAS formation (based on Atg1 and Atg13 puncta) was downregulated in Rpb9-depleted cells, but could be rescued by exogenous expression of HA-Rpb9 (Fig. EV2G, H). We examined Atg9 recruitment to Atg8 puncta, and the results showed that Atg9 recruitment was not affected by Rpb9 deletion (Fig. EV2I). We observed the membrane elongation process by examining Atg12-Atg5 conjugation, and the results showed that Atg12-Atg5 conjugation was not affected by Rpb9 deletion (Fig. EV2J). To examine whether Rpb9 affects autophagosome-vacuole fusion, we observed Atg8 puncta, as

disruption of the fusion process would cause accumulation of autophagosomes in cells, as shown by the increased number of Atg8 puncta. The results indicated that Rpb9 does not function in the fusion process, as its deletion causes a diffuse distribution of Atg8 (a phenotype similar to Atg1 deletion but different from Vam3 deletion), which caused fusion to fail; thus, Atg8 puncta were observed in cells (Fig. 2D, E). Unfortunately, we failed to detect the phosphorylation status of Atg1 and Atg13, although we tried many times using Phos-tag™ SDS–PAGE (WAKO company), which was specifically designed for the detection of phosphorylated proteins. Radioactive labeling of phosphorylated proteins was also unavailable in our current situation.

By examining the different processes of autophagy affected by Rpb9, the above results suggest that Rpb9 regulates autophagosome formation instead of fusion. Furthermore, these results indicate that Rpb9 regulated PAS formation (mainly by Atg1, Atg13 and Atg17). Combined with other results that showed that the mRNA and protein levels of Atg1 but not Atg13 or Atg17 were affected by Rpb9 (Fig. EV3B, C), we speculated that Rpb9 functions in autophagy by regulating ATG1 transcription.

2) "*(2) Related to Figures 1G and 3D, protein expression levels of Atg1 (and other Atg proteins such as Atg13 and Atg17) should be investigated, because there is no data showing the relationship between the transcription level and translation level of Atg1 in WT and rpb9Δ cells.*"

Response: We thank the referee for this suggestion. As suggested, we detected the protein levels of Atg1, Atg13 and Atg17 before and after starvation in WT and *rpb9*Δ cells. Related to Figure 1G, we analyzed the protein levels of Atg1, Atg13 and Atg17 after starvation, and the results showed that Atg1 was induced by starvation, but Atg13 and Atg17 were not (Fig. EV1A). Related to Figure 3D, we analyzed the protein levels of ATG1, ATG13 and ATG17 in WT and *rpb9*Δ cells before and after starvation, and the results showed that the protein levels of Atg1, but not Atg13 or Atg17, were specifically increased upon starvation, depending on Rpb9 (Fig. EV3C).

3) "*(3) In Figure 2A, both experimental conditions and results of the high-throughput screen are not shown. This reviewer wondered whether all of 4857 non-essential genes were examined or only transcriptional compontns were examined?"*

Response: The authors apologize for the lack of clarity. The screen was performed on 4857 nonessential genes. After screening, 673 strains selected on the basis of the starvation-resistance phenotype were analyzed; we focused on potential transcription-regulating factors, which led to the discovery of Rpb9 as a potential regulator of autophagy.

For the screen performance, the knockout library (4857 nonessential genes) strains were grown on YPD plates followed by starvation on nitrogen-deficient plates for 7 days. After starvation, the yeast cells were regrown on YPD plates to assess their viability. The potential candidates from this screen were selected based on observation of starvation resistance (because deletion of an autophagy gene causes cell death after starvation).

This information on the screening procedure was added to the manuscript (figure legends). The list of genes in the knockout library (4857 nonessential genes) and the list of selected genes after screening are shown in Dataset EV2.

4) "*(4) In Figure 2B etc., the authors used GFP-50Q as an autophagy reporter, however, the GFP-50Q reporter may be a "selective" substrate. If GFP-50Q is a selective autophagy reporter, the defects observed in rpb9Δ cells also indicate the defects in selective autophagy, not normal autophagy. GFP-Atg8 or Pgk1-GFP should be used to investigate normal autophagic activity. Alternatively, appropriate references showing that GFP-50Q is a normal autophagy reporter should be cited. (Related to the minor comment (3), this reviewer could not know the detail of the 50Q construct.)"*

Response: We apologize for not presenting this information more clearly. The lists of yeast strains and plasmids were provided in the Supplementary tables in the initial submission. However, they were not integrated with the main manuscript for review. We apologize for this inconvenience. In the revised manuscript, the strain list and plasmid list are shown in Table EV1 and Table EV2, providing detailed information on the yeast strains and plasmids.

Following the referee's suggestions, we cited references showing GFP-50Q as a normal autophagy reporter (Ref. Shen et al, 2021-PMID: 34788606; Zhang et al, 2021-PMID: 32693712). Furthermore, we used GFP-Atg8 and Pgk1-GFP to investigate the autophagic activities in wild-type cells and *rpb9*Δ cells. The results showed that the autophagic degradation of GFP-Atg8 and Pgk1-GFP was deficient in *rpb9*Δ cells (Fig. EV2A, B).

5) "*(5) In Figure 5, EMSA using both Rpb9 and Gcn4 should be examined, because there is no direct evidence indicating the Rpb9 binding to the promoter region of the ATG1 gene."*

Response: We thank the referee for this suggestion. Indeed, EMSAs using both Rpb9 and Gcn4 proteins are important to support the conclusion that Rpb9 binds to the *ATG1* promoter (through Gcn4). We performed EMSAs using His-tagged Rpb9 and GST-tagged Gcn4 purified from *E. coli* cells together with DNA probes spanning the promoter regions of *ATG1*. The results showed that Rpb9+Gcn4 binds the -880 bp and -760 bp regions of the *ATG1* promoter but not the -350 bp region (Fig. 5B). Together, the results showed that the Rpb9 protein alone could not bind the *ATG1* promoter (Fig. EV4B) and Rpb9 directly interacts with Gcn4 (Fig. 5E); it was thus proposed that Rpb9 binds the *ATG1* promoter through Gcn4.

6) "*(6) In Figure 6G, autophagic activities should be examined in POLR2I knockdown mammalian cells. The Rpb9 orthologs and transcriptional regulation of the ATG1/ULK1/3 gene were conserved, but its importance was not elucidated."*

Response: We thank the referee for this suggestion. We analyzed the protein levels of the autophagy receptor p62 and the number of LC3 puncta in POLR2I knockdown mammalian cells. p62 recruits cargos into autophagosomes for autophagic degradation, and p62 itself is also degraded in this process. Thus, the protein levels of p62 reflect its autophagic degradation and could be analyzed to detect autophagy activities. The results showed that the p62 protein level was increased and the autophagosome number (shown by LC3 puncta) was reduced in POLR2I knockdown mammalian cells (HEK293T cells) (Fig. EV5C, D). This suggested the importance of the human homolog of yeast Rpb9, POLR2I, in regulating autophagy in mammalian cells.

7) "*(7) In Figure 1F, please explain how to validate the results of the mRNA expression sequencing."*

Response: We are uncertain whether this point is asking us to explain how the genome mRNA expression sequencing was performed or how the validation of the sequencing data was performed. Regardless, we added information about how the genome mRNA expression sequencing was performed in the Materials and Methods section "Genome mRNA expression sequencing". The results of the mRNA expression sequencing showed that *ATG1* was the most upregulated among the autophagy genes (Fig. 1G). We validated and confirmed this by analyzing the transcription of *ATG* genes (*ATG1-ATG18*) by quantitative real-time polymerase chain reaction (qRT–PCR) in yeast cells before and after starvation, demonstrating the significant upregulation of *ATG1* compared with other *ATG*s (Fig. 1H). The qRT–PCR methodology information is described in the Materials and Methods section "RNA extraction and quantitative real-time PCR (qRT–PCR)".

Minor comments:

8) "*(1) In Figure 1D, it is difficult to understand the experimental conditions. Does "R -> T" mean "R (3 h, 2nd lane) -> R+T (13 h, 3rd lane)"? If so, why the GFP processing efficiencies are so different between Figure 1D (2nd lane) and Figure 1B (2nd lane). If "R (3 h, not shown) -> R+T (3 h, 2nd lane; 16 h, 3rd lane)" is correct, the "R (3 h)" data should be shown. Experimental conditions should be described in detail.*"

Response: We apologize for this lack of clarity. The referee's second speculation is correct regarding this experimental design. First, the yeast cells were induced for 3 h with rapamycin (R) or SD-N starvation (S), and then the transcription inhibitor thiolutin was added for another 3 h or 13 h. The scheme of this experimental design was modified (Fig. 1C), and experiments were repeated with R or S for 3 h (sample 3 h) and R/S+T for 3 h and 13 h (samples 6 h and 16 h). The results showed that a short induction time (3 h) was sufficient to activate autophagy, and pretreatment with a transcription inhibitor effectively blocked autophagy (Fig. 1D, E).

9) "*(2) In Figure 2D, this reviewer could not understand why vam3Δ and ypt7Δ were used. What do the authors mean by these results? Rather, exogenous expression of HA-Rpb9 should be included in Figure 2D, 2E, 2F, and 3D as well as Figure 2B.*"

Response: The *vam3*Δ and *ypt7*Δ cells were used as controls to show the accumulation of Atg8 puncta (autophagosomes) in yeast cells with a deficiency of proteins, which function in autophagosome fusion. The Atg8 distribution phenotype of *rpb9*Δ cells was similar to that of *atg1*Δ cells and different from that of *vam3*Δ cells (Fig. 2D, E), suggesting that Rpb9 functions in autophagosome formation instead of fusion.

As suggested, we repeated the previous Fig. 2D, 2E, 2F, and 3D experiments, including exogenous expression of HA-Rpb9. The results showed that exogenously expressed HA-Rpb9 recovered autophagy (Fig. 2D, F, G and Fig. EV2C-E) and *ATG1* transcription (Fig. EV3B) in *rpb9*Δ cells.

10) "*(3) This reviewer could not find the constructs of the HA-Atg1 expression plasmid, etc. and the 50Q construct. What vector was used and what promoter was used?*"

Response: We apologize for not presenting this information clearly. The yeast strain list and plasmid list were provided in the Supplementary tables in the initial submission. However, they were not integrated with the main manuscript for review. We apologize for this inconvenience. In the revised manuscript, the strain list and plasmid list are shown Table EV1 and Table EV2, showing detailed information on the yeast strains and plasmids.

For the HA-Atg1 expression plasmid, the vector p413-ADH was used, and the promoter was prADH1. For the GFP-50Q plasmid, the pUG36 vector was used, and the promoter was prMET25.

11) "*(4) In Figure 2H, why the GFP moieties are not detected?*"

Response: In Figure 2H (now Fig. 2I), Cpy1-GFP transfer into the vacuole was observed. Cpy1 is an exopeptidase involved in protein degradation in the vacuole. Cpy1 remains intact in vacuoles, similar to the GFP moiety, which resists vacuole degradation. Therefore, Cpy1-GFP remains a fused protein

without the GFP moiety generated. In contrast, the autophagy substrate (GFP-Atg8) and endocytosis substrate (GFP-Sna3) are degraded in vacuoles, leading to the generation of GFP moieties (Fig. 2H).

12) "*(5) In Figure legend 2, (D) in line 626 is (E). The same applies thereafter.*"
Response: We apologize for this mistake. We thank the referee and have corrected the Figure 2 legend.

Referee #2

We thank the referee for the time and positive comments "***At present, most of the studies on ATG are on their post-translational modifications (PTMs) and thus this study reveals a new window in study of autophagy mechanisms.***". The specific and helpful comments are addressed as shown below.

Major comments:

1) "*1. Regarding the inhibitory effect of CHX on autophagy, the authors need to be more cautious on their conclusions, based on the fact that CHX has indirect effect on autophagy by increasing the intracellular AA concentration and subsequent activation of mTORC1. Therefore, the authors need to use additional method with additional data to support the conclusion that "autophagy induction depends on the synthesis of new proteins".*"
Response: We thank the referee for this suggestion. Indeed, CHX exerts multiple effects on autophagy in addition to its function in blocking Atg protein translation. In addition to the transcription inhibitor (Fig. 1B), we performed new experiments inducing Atg1 expression to support the conclusion that "autophagy induction depends on the synthesis of new proteins".

Because ATG1 is the most upregulated factor among ATGs (Fig. 1G), we constructed Atg1 expression plasmids using an inducible GAL1 promoter (Fig. EV1B). prGAL1-HA-ATG1 was transformed into *atg1*Δ yeast cells cultured in a

medium supplemented with raffinose as a sugar source; therefore, the expression levels of HA-Atg1 were similar to those of endogenous Atg1 (chromosomally C-terminal HA-tagged Atg1) (Fig. EV1C). When starved in a nitrogen-deficient medium supplemented with galactose, the prGAL1-HA-ATG1 plasmids induce high levels of Atg1, and autophagy was found to be maintained, as shown by the degradation of GFP-Atg8 (Fig. EV1D). However, when starved in nitrogen-deficient medium supplemented with raffinose, the prGAL1-HA-ATG1 plasmids maintained low levels of Atg1, and autophagic degradation of AGP-Atg8 was blocked (Fig. EV1D). Based on these results together with the observation that the translation inhibitor CHX and the transcription inhibitor thiolamine blocked autophagy induction (Fig. 1A, B), we proposed that autophagy induction depends on the synthesis of new proteins.

2) "*2. Figure 1: Authors also need to show the changes of Atg1 protein level, in corresponding to the changes of Atg1 mRNA level."*

Response: We thank the referee for this suggestion. As suggested, we determined the protein levels of Atg1 before and after starvation. The results showed that the protein levels of Atg1 were induced by starvation, but those of Atg13 and Atg17 were not (Fig. EV1A), which is consistent with the induced mRNA levels of Atg1 (Fig. 1G, H).

3) "*3. Figure 3C: In addition to Atg1, several other Atgs such as Atg5 and Atg13 were also down-regulated in rpb9 KO cells. On the other hand, the authors found that only Atg1 overexpression, but not Atg9, Atg13, and Atg17, can restore the defective autophagy in rpb9 KO cells. Here the authors need to address the following points: (i) Is rpb9 also implicated in the transcriptional regulation of Atg5 and Atg13? (ii) can the authors show the protein level of various Atg protein in the over-expression experiments? And (iii) can the authors test the effect of Atg5 which is also down-regulated?"*

Response: We thank the referee for these suggestions.

(i) We determined the mRNA levels of Atg5 and Atg13 by quantitative real-time polymerase chain reaction (qRT–PCR). The results showed that the mRNA levels of Atg5 were not affected by Rpb9 deletion and that the mRNA

levels of Atg13 were very slightly affected by Rpb9 deletion (Fig. EV3B). Therefore, we speculated that Rpb9 had little or no effect on the transcriptional regulation of Atg5 and Atg13.

(ii) We included the original blots showing the protein levels of various Atg proteins in the overexpression experiments (Fig. 3E-H).

(iii) The effect of Atg5 overexpression in rpb9 KO cells was analyzed. Similar to Atg1, Atg9, Atg13 and Atg17, Atg5 was overexpressed in *rpb9*Δ cells, and autophagic degradation of GFP-50Q was detected. The results showed that Atg5 overexpression could not rescue autophagy in *rpb9*Δ cells (Fig. 3I).

4) "*4. Figure 5F: By examining the data carefully, actually over-expression of Gcn4 can partly restore autophagy in rpb9 KO cells, evidenced by the weak but obvious GFP band. In contrast, over-expression of rpb9 in Gcn4 KO cells failed to have the same effect, (Figure 5G). In both the text and figure legend, the authors failed to describe this difference."*

Response: We thank the referee for this reminder. Indeed, overexpression of Gcn4 partly restored the autophagic degradation of GFP-50Q in *rpb9*Δ cells (Fig. 5F), whereas overexpression of Rpb9 in *gcn4*Δ cells failed to have the same effect (Fig. 5G). Together with the results showing that Rpb9 only binds the ATG1 promoter in the presence of Gcn4 (Fig. 5B and Fig. EV4B), we speculated that Gcn4 binds the ATG1 promoter and then interacts with Rpb9; the latter recruits the RNA polymerase II complex by interacting with the Rpb1 and Rpb2 subunits. There is a possibility that Gcn4 directly and weakly binds the RNA polymerase II complex in the absence of Rpb9 or that unknown factors other than Rpb9 exist to recruit RNA polymerase II to ATG1 promoter-bound Gcn4. As suggested, a short description of this difference between Gcn4 overexpression and Rpb9 overexpression and a summary of the above speculation have been added to the revised manuscript when introducing the results of Fig. 5F, G.

5) "*5. Figure 5H: The model shown in this panel does not really reflect the data presented in the study, based on the comments in Point #4 above."*

Response: We agree with the referee. We deleted this model scheme in the revised manuscript.

Referee #3

We thank the referee for the time and efforts. Comments and suggestions are very helpful for improving this work. We have studied the comments carefully and addressed accordingly, as shown below.

Major Points:

1) "*The authors should compare the Atg1 protein levels in nutrient-rich and nitrogen starvation medium (e.g. 3, 6 and 16 hours) for wild type and Rpb9 deleted yeast to show how changes in mRNA levels translate into changes in protein expression levels?"*

Response: We thank the referee for this suggestion. We detected the protein levels of Atg1 before and after starvation in WT and Rpb9-deleted yeast cells. The results showed that the protein levels of Atg1 were induced by starvation, but Atg13 and Atg17 were not (Fig. EV1A), which is consistent with the induced mRNA levels of Atg1 (Fig. 1G, H). The protein levels of Atg1 in Rpb9-deleted cells were reduced compared with those in WT cells (Fig. EV3C), which is consistent with the observation that the mRNA levels of Atg1 were reduced by Rpb9 deletion (Fig. 3D).

2) "*The authors should also study selective autophagy under nutrient-rich conditions (e.g. the Cvt pathway) in wild type and Rpb9 deleted cells."*

Response: We thank the referee for this suggestion. Selective autophagy of Ape1 (the autophagic substrate of the Cvt pathway) was examined in wild-type and Rpb9-deleted cells under nutrient-rich conditions. The results showed that autophagic transfer of Ape1 into vacuoles (as indicated by maturation of prApe1 into mApe1) was blocked by Rpb9 deletion (Fig. EV2F).

3) "*To validate their model (Figure 5H) the authors should repeat the pulldown shown in Figure 5E with recombinant Rpb9 to demonstrate a direct interaction."*

Response: We thank the referee for this suggestion. We performed GST pulldown assays using His-tagged Rpb9 and GST-tagged Gcn4 purified from *E. coli* cells. The results showed that GST-Gcn4 could pull down His-Rpb9, whereas GST could not (Fig. 5E), suggesting that the interaction between Gcn4 and Rpb9 is direct.

Minor Points:

4) "*Figure 2H*

The CPY1-GFP cleavage assay lacks a negative control. Can the author please include a strain with defective CPY processing?"

Response: We thank the referee for this suggestion. We included control strains with defective CPY processing, *vps15*Δ and *vps34*Δ, as Vps15 and Vps34 were reported to be necessary for CPY processing (J Cell Biol. 2001 Feb 5;152(3):519-30.). The results showed that CPY could not be transported into vacuoles in *vps15*Δ and *vps34*Δ cells (Fig. EV2K).

5) "*Figure 2E*

Ape1 should also be transported to the vacuole under nutrient-rich conditions. Hence, why can Ape1 not be found in the vacuole under those conditions?"

Response: We speculated that endogenous Ape1 is indeed transported to the vacuole, becoming the mature form under nutrient-rich conditions, as shown in the literature and our results (Fig. EV2F). However, exogenous fluorescently tagged Ape1 exhibited a cytoplasmic puncta distribution and was transported into vacuoles for maturation upon starvation or rapamycin treatment, as shown by our results (Fig. 2F) and in the literature (e.g., Fig. 1E in Proc Natl Acad Sci USA. 2009 Oct 6,106(40):17049-54; Fig. 2C in Autophagy. 2011 Jul, 7(7):716-26; Fig. 5C in Mol Cell. 2014 Feb 6, 53(3):471-83; and Fig. 1a in Nat Commun. 2021 Dec 10, 12(1):7194).

6) "*The authors should repeat the experiments using a strain with untagged Ape1 and monitor Ape1 processing by Western blotting.*"

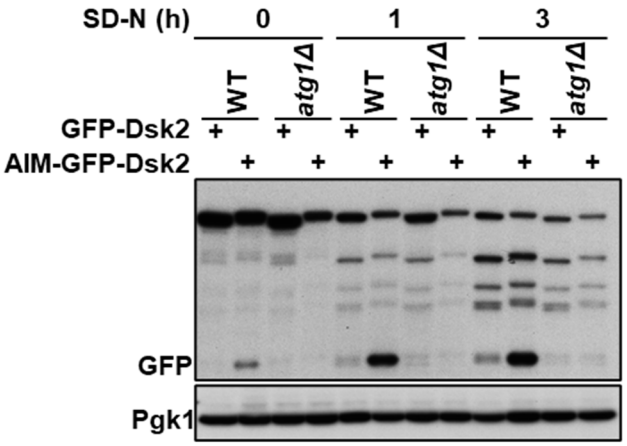
Response: We thank the referee for this suggestion. We monitored the processing of endogenous Ape1 by western blotting, and the results showed that the processing of untagged endogenous Ape1 (indicated by maturation of prApe1 into mApe1) was blocked by Rpb9 deletion (Fig. EV2F).

General:

7) "*With the Atg1 protein expression levels in mind can the authors speculate in the discussion why the Atg1 expression levels in cells grown in nutrient-rich medium are sufficient to promote selective autophagy pathways such as the Cvt pathway but not bulk autophagy?*"

Response: We thank the referee for this suggestion. Autophagy activities are at low levels because of the low levels of Atg1 and other Atg proteins in yeast cells grown in nutrient-rich medium. Such low levels of autophagy can facilitate the Cvt pathway (the autophagic transport of Ape1 into vacuoles) but cannot induce the degradation of bulk autophagy substrates. We speculate that the reason for this is the nature of the substrate. The Cvt substrate prApe1 forms a special dodecameric architecture, and disruption of this dodecameric assembly prevents vacuolar targeting (Autophagy. 2015;11(9):1580-93.). Therefore, the nature of prApe1 makes it a very suitable substrate for autophagosome targeting (with the help of Atg11 and Atg19).

However, a substrate protein that is not subject to autophagic degradation under nutrient-rich conditions can also undergo autophagic degradation. Several years ago, when we analyzed the pathway determinants of proteasome receptors and autophagy receptors, we found that adding Atg8-interacting motif (AIM) to proteasome receptor Dsk2 caused its autophagic degradation even under nutrient-rich conditions (shown in the results below). After autophagy activation upon starvation, the autophagic degradation of AIM-Dsk2 is clear (shown in the results below).



In conclusion, we speculate that the reason why low Atg1 levels under nutrient-rich conditions support the Cvt pathway (autophagic transport of prApe1) is that prApe1 is a very suitably organized protein cargo, which facilitates its autophagosome targeting (with the help of receptors Atg19 and Atg11), even at very low levels of autophagic activity. For the degradation of bulk autophagy substrates, higher autophagic activity is needed. When a special feature such as Atg8 targeting (through AIM) is added to a bulk autophagy substrate, it can also be subject to autophagic degradation under low autophagy conditions (nutrient-rich medium). Of course, this is just the authors' speculation; it is possible that there are other reasonable explanations for these observations.

8) "*Can the authors show where the first zinc finger domain and the linker region in Rpb9 responsible for its discussed function are located with respect to the RNA polymerase?"*

Response: We thank the referee for this suggestion. Based on the structure of RNA polymerase II (Ref.), we presented a schematic diagram showing the first and second zinc finger domains and the linker region of Rpb9 in RNA polymerase II (Fig. EV4A).

9) "*In yeast as opposed to mammalian cells there is no bulk autophagy under nutrient-rich conditions. The authors should adjust the introduction accordingly."*

Response: We thank the referee for this suggestion. We adjusted the introduction accordingly as shown below.

“In mammalian cells, autophagy occurs at low basal levels under normal conditions, and under stress conditions, such as nutrient deficiency, hypoxia, and pathogen infection, autophagy is rapidly and dramatically upregulated(Levine & Kroemer, 2019; Jin et al, 2014; Kawabata & Yoshimori, 2020). In yeast, bulk autophagy is inhibited under nutrient-rich conditions. A special type of selective autophagy, the cytoplasm-to-vacuole targeting (Cvt) pathway, occurs in yeast cells when external nutrients are available. The Cvt pathway selectively targets and transfers cytoplasmic hydrolase proteins, including Ape1, Ams1 and Ape4, to the vacuole(Yuga et al, 2011; Harding et al, 1995; Hutchins & Klionsky, 2001; Klionsky et al, 1992). The Cvt pathway is biosynthetic rather than degradative, as hydrolases are transported into vacuoles for biological functions instead of being degraded(Baba et al, 1997). Cvt cargos are recruited into a special type of autophagosome, which is usually smaller (approximately 150 nm) than starvation-induced autophagosomes (approximately 500 nm)(Mizushima & Klionsky, 2007; Sawa-Makarska et al, 2014). The Cvt pathway requires the common core autophagic machinery and specific receptors Atg19 and Atg11(Sawa-Makarska et al, 2014; Yorimitsu & Klionsky, 2005). ”.

Dear Dr. Lu,

Thank you for the submission of your revised manuscript. We have now received the enclosed comments from the referees.

As you will see, referee 3 still has one more comment that needs to be addressed before we can proceed with the acceptance of your manuscript here.

A few other editorial requests will also need to be addressed:

- The use of the Data Availability Section (DAS) is incorrect. In this paragraph, links to data deposited in public databases should be listed (e.g. sequencing data or else). If you have not deposited any data in public databases, please mention this fact in the DAS.

- Please remove the author credits from the manuscript file.

- The reference format needs to be corrected: Not more than 10 authors may be listed, and the issue number should not be in bold. EMBO reports uses the Harvard reference style.

- I attach to this email a manuscript file with comments by our data editors. Please address all comments in the final ms.

I would like to suggest some changes to the manuscript title and abstract that needs to be written in present tense. Please let me know whether you agree with the following:

The RNA polymerase II subunit Rpb9 activates ATG1 transcription and autophagy

Macroautophagy/autophagy is a conserved process in eukaryotic cells that mediates the degradation and recycling of intracellular substrates. Proteins encoded by autophagy-related (ATG) genes are essentially involved in the autophagy process and must be tightly regulated in response to various circumstances, such as nutrient-rich and starvation conditions. However, crucial transcriptional activators of ATG genes have remained obscure. Here, we identify the RNA polymerase II subunit Rpb9 as an essential regulator of autophagy by a high-throughput screen of a Saccharomyces cerevisiae gene knockout library. Rpb9 plays a crucial and specific role in upregulating ATG1 transcription, and its deficiency decreases autophagic activities. Rpb9 promotes ATG1 transcription by binding to its promoter region, which is mediated by Gcn4. Furthermore, the function of Rpb9 in autophagy and its regulation of ATG1/ULK1 transcription are conserved in mammalian cells. Together, our results indicate that Rpb9 specifically activates ATG1 transcription and thus positively regulates the autophagy process.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a final version of your manuscript as soon as possible. Please let me know if you have any questions or comments.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

I have reviewed the revised manuscript and the point-by-point response to my previous comments. All my concerns have been appropriately addressed and the added data strengthens the authors' new findings that Rpb9 regulates ATG1 transcription by binding to the ATG1 promoter region and that deletion of RPB9 causes defects in autophagosome formation in the initial step of Atg1 function. I recommend publication of this work in EMBO Reports.

Referee #2:

The authors have made a great deal of efforts in addressing reviewers' comments by performing new experiments and addition of new data, without corresponding changes of the text. Overall, I have no further concerns with this revised MS and thus support the publication at EMBO Report.

Referee #3:

One of the major points raised by all reviewers was whether the Rpb9-dependent change in Atg1 mRNA levels would also translate into changes in Atg1 protein levels. The authors have attempted to address this critical point, however, as the Western blot results (Figures EV1A and EV3C) are not quantified and the changes very subtle with the increase in Atg1 protein levels upon starvation worryingly similar in both WT and in rpb9 deleted cells (again this needs to be quantified) suggesting possible other Rpb9 independent mechanism(s), it would be premature to accept the study already at this stage.
These inconsistent results may, however, be due to the suboptimal experimental set up the authors chose. The authors should carry out the same experiments shown in Figures EV1A and EV3C in a ypt7 deletion background as Atg1 is transported to the vacuole and degraded in cells undergoing autophagy due to its Atg8 interacting motifs and resultant phagophore association and non-specific bulk autophagy dependent uptake. Hence, an Rpb9 dependent increase in Atg1 levels may be obscured by its simultaneous degradation in WT but not rpb9 deletion cells.

If would fully support publication of this study if the authors could address this key point.

Responses to the Referees

We sincerely thank all the three referees for their appreciation of our revised manuscript. The remaining concern from referee 3 is clearly expressed, and helpful suggestion is also kindly provided. We have addressed this issue and revised the manuscript accordingly.

Referee #3

"*One of the major points raised by all reviewers was whether the Rpb9-dependent change in Atg1 mRNA levels would also translate into changes in Atg1 protein levels. The authors have attempted to address this critical point, however, as the Western blot results (Figures EV1A and EV3C) are not quantified and the changes very subtle with the increase in Atg1 protein levels upon starvation worryingly similar in both WT and in rpb9 deleted cells (again this needs to be quantified) suggesting possible other Rpb9 independent mechanism(s), it would be premature to accept the study already at this stage.*

These inconsistent results may, however, be due to the suboptimal experimental set up the authors chose. The authors should carry out the same experiments shown in Figures EV1A and EV3C in a ypt7 deletion background as Atg1 is transported to the vacuole and degraded in cells undergoing autophagy due to its Atg8 interacting motifs and resultant phagophore association and non-specific bulk autophagy dependent uptake. Hence, an Rpb9 dependent increase in Atg1 levels may be obscured by its simultaneous degradation in WT but not rpb9 deletion cells.

If would fully support publication of this study if the authors could address this key point.".

Response: We thank the referee 3 for this suggestion. As suggested, we analyzed the protein levels of Atg1 before and after starvation in *ypt7*Δ background yeast cells. Results showed that Atg1 protein levels were obviously induced by starvation (Fig. EV1B, western blots and quantification). Rpb9 was deleted in *ypt7*Δ cells and the protein levels of Atg1 were detected. Results showed that the protein levels of Atg1 in *ypt7*Δ cells were obviously increased upon starvation but not in *ypt7*Δ *rpb9*Δ cells (Fig. EV3D, western blots and quantification), indicating that the upregulation of Atg1 protein levels is dependent on Rpb9.

Dr. Kefeng Lu
Sichuan University
Renminsouth Sanduan 17
Chengdu, Sichuan 610041
China

Dear Dr. Lu,

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,

Esther Schnapp, PhD
Senior 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

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-2022-54993V3 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: Kefeng Lu	
Journal Submitted to: EMBO Reports	
Manuscript Number: EMBOR-2022-54993V1	

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	Materials and Methods

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 Methods

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.	Not Applicable	

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.	Not Applicable	
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.	Not Applicable	

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.	Yes	Materials and Methods

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.	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.	Yes	Figure Legends
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? 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?	Not Applicable	

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?	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	