

Damage-induced regeneration of the intestinal stem cell pool through enteroblast mitosis in the *Drosophila* midgut

Aiguo Tian, Virginia Morejon, Sarah Kohoutek, Yi-Chun Huang, Wu-Min Deng, and Jin Jiang

DOI: 10.15252/emboj.2022110834

Corresponding author: Aiguo Tian (atian@Tulane.edu)

Review Timeline:

Submission Date:	28th Feb 22
Editorial Decision:	4th Apr 22
Revision Received:	14th Jun 22
Editorial Decision:	12th Jul 22
Revision Received:	14th Jul 22
Accepted:	15th Jul 22

Editor: Daniel Klimmeck

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 Tian,

Thank you again for the submission of your manuscript (EMBOJ-2022-110834) to The EMBO Journal. Your study has been sent to three reviewers with *Drosophila* stem cell-developmental expertise, however one reviewer got much delayed and, has not sent us his/her report so far. We have received feedback from the other two referees, which I enclose below, and decided to - in the interest of the timeliness of the data - proceed with our decision based on these reports.

As you will see, the referees acknowledge the potential interest and novelty of your results, although they also express a number of major issues that will have to be conclusively addressed before they can be supportive of publication of your manuscript in The EMBO Journal. In more detail, referee #1 states that better characterisation of mitotic EBs and their coordination with regular ISC proliferation is required in order to strengthen the claims made (ref#1, pts.2,5). Further, this reviewer points to concerns on evidence provided for enteroblast mitotic re-entry as a physiological phenomenon (ref#1, pt.4), and requests complementary investigation to clarify the relative contribution of EGFR-Ras over other pathways in this conversion (ref#1, pt.3). Referee #2 points to important issues with on the robustness of the results and asks you to consolidate the findings by more marker stainings and drivers (ref#2, pt.1, see also ref#1, pts.1,2). In addition, the reviewers raise a number of points related to additional controls required, overall data discussion and literature references that would need to be conclusively addressed to achieve the level of robustness and clarity needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments, pending there are no major concerns by referee #3 on the technical robustness of the work.

I will share the report of this expert as soon as we receive it.

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

When submitting your revised manuscript, please carefully review the instructions below.

Thank you for the opportunity to consider your work for publication.
I look forward to your revision.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

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 (3rd Jul 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

Referee #1:

Tian et al. report new and important insights regarding enteroblast (EBs)-mitosis in the *Drosophila* midgut. Since instances of EB-mitosis have been reported previously, it is interesting to uncover the mechanism of this process. I recommend publication of the manuscript after a few additional experiments are added to strengthen the model.

1) In Figure 2, su(H)>GFP guts GFP+PH3+ cells are 100% without DI (DI-). This is an important experiment that becomes the foundation for the overall model. However, the use of only one driver weakens the model. Since EB drivers are lacking, the

authors could strengthen their point by using an ISC driver like the *esgGal4,su(H)-Gal80* or *DI-Gal4* together with a destabilized-GFP and assay for non-GFP, non-DI but PH3+ cells (GFP-PH3+DI-) and quantify them.

2) Moreover, the authors report the existence of EB-mitotic cells but do not really characterize them except for reporting that they are DI-. How different are they from regular EBs? Do they express Sox21a? Is *piezo* irregularly expressed in them? Do they have higher *esg* expression and therefore higher stemness potential? Or higher Notch expression than regular EBs? Also, how close are they to the basal membrane? Do they express more integrins? And therefore, could more integrins combined with closeness to the basal membrane influence them to divide? Since this study is about the identification of a functionally different subpopulation of EBs, the authors should characterize these cells by providing a set of additional markers to distinguish mitotic-EBs from non-mitotic EBs.

3) The authors propose that this is a Ras/EGFR-dependent process. However, the authors do not test any other proliferative pathway. How specific is EB-mitosis to Ras/EGFR signaling? Can reduction of Hippo or induction JNK or JAK/STAT or Wnt also induce EB mitotic divisions. This is an important point to address that will be of interest to the whole intestinal regenerative field in *Drosophila*.

4) The authors use the centromere UAS-Cnn-GFP marker or reduce mitosis with *stg*-RNAi under *Su(H)*>Ras induction. Since the premise of this paper is that EB-mitosis is a physiological phenomenon, the authors should use those tools during infection.

5) Finally, how dependent is EB-mitosis to ISC-mitosis? Is EBs-mitosis a compensatory mechanism for ISC proliferation? For example, if the authors after inducing infection (after proliferation has started) prevent temporally ISCs from dividing (e.g. *DITS*>*stg*RNAi+ destabilizedGFP) and assay for mitotic-EBs (GFP-PH3+) how will those EBs behave? Will the levels of EB-mitosis decrease because ISC division is reduced? or will they increase and therefore act as a compensatory mechanism?

Referee #2:

The paper by Aiguo TIAN and colleagues reports a nice work concerning the control of the intestinal stem cell division in the response to infection. Briefly, it has been well established that the adult *Drosophila* intestinal stem cells (ISCs) produce post-mitotic progenitors called enteroblasts (EBs), which will differentiate into enterocytes (ECs). Here, the authors demonstrate that upon gram-negative bacterial infection EBs are able to divide and produce cells bearing the features of ISCs. They further show that this "dedifferentiation" requires EGF-R activation and that activation of the pathway in EBs is sufficient to trigger their division in ISCs.

The experimental design is compelling, the experiments appear well done, and their results are interpreted rigorously. The text is well written and the figures properly presented.

I believe that this work provides a significative shift in our understanding of the ISC lineage and should interest many colleagues in the field.

Here are some suggestions to improve the manuscript:

Major points:

1) Although the experimental plan is pretty complete, the main conclusion of the paper relies on using a single *Gal4*-driven line (*Su(H)-Gal4*), as well as a single marker of ISCs (*Delta*). For example, *Delta* is also expressed in pre-EEs. Additional markers and ideally driver(s) would ascertain the taking home message of the study.

2) Recent work has shown that the other type of ISC-derived progenitors, called pre-EEs and that will differentiate into Enteroendocrine cells (EEs), are also able of one round of division (e.g., Chen et al., *Nat Cell Biol* 2018). I guess it would be fair to introduce this notion, including in Fig 1A.

Minor points:

Line 45-46: Work from the Perrimon lab using single cell RNA-seq (Hung et al., *PNAS* 2020) has provided evidence of the unsuspected diversity of *Drosophila* ECs and EEs, and revealed some populations exhibiting molecular signatures similar to Paneth cells, and to tuft and goblet cells, respectively.

Line 108: I guess that dividing EBs should be Ph3+ and GFP+?

Line 139: The non-cell-autonomous effects seen upon overexpression of EGF_r ligands in EBs makes good sense. In contrast, how the activation of the EGF-RECEPTOR in EBs can lead to a similar phenotype is less clear. The authors mentioned in the discussion that EGF-r activation might be relayed by the JAK-STAT pathway. Have they evidence for that in EBs?

Line 235: If I'm right, pre-EEs are also Prospero-positive. Do the authors have additional evidence for EB progeny can produce terminally differentiated EEs? If not, the pre-EE fate should be mentioned as well.

Dear Dr. Klimmeck,

We have revised our manuscript entitled “Regeneration of intestinal stem cells through enteroblast mitosis in *Drosophila* midguts” based on the reviewers’ comments. On behalf of all authors, we would like to thank all reviewers for taking time to give us valuable and constructive comments on our manuscript. We have carefully revised our manuscript and addressed all questions raised by the reviewers. We think their constructive suggestions contributed greatly to the improvement of our manuscript, and we hope that the reviewers will be able to support the publication of our revised MS in EMBO J.

The revised manuscript includes additional experiments as requested by the reviewers. We added new figures and panels (Fig 2D-G, Fig 3, Fig 5L-N, Fig EV2, Fig. EV5, Fig. EV6 and Fig. EV7), and updated existing figures (Fig 1A). We have highlighted these changes in the main text.

Below you will find our point-by-point response to all comments from reviewers

Reviewer 1

Since instances of EB-mitosis have been reported previously, it is interesting to uncover the mechanism of this process. I recommend publication of the manuscript after a few additional experiments are added to strengthen the model.

We thank the reviewer for these positive comments.

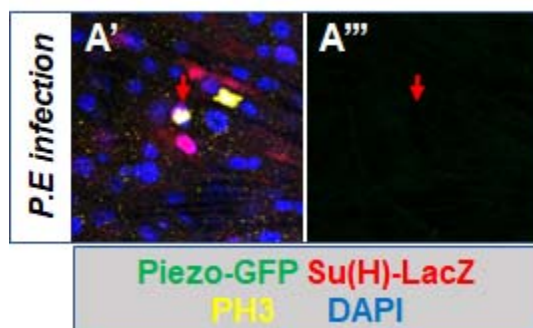
1) In Figure 2, su(H)>GFP guts GFP+PH3+ cells are 100% without DI (DI-). This is an important experiment that becomes the foundation for the overall model. However, the use of only one driver weakens the model. Since EB drivers are lacking, the authors could strengthen their point by using an ISC driver like the *esgGal4*, su(H)-Gal80 or DI-Gal4 together with a destabilized-GFP and assay for non-GFP, non-DI but PH3+ cells (GFP-PH3+DI-) and quantify them.

We performed an experiment with *esgGal4*, su(H)-Gal80>UAS-GFP to mark ISCs. In this experiment, the female flies expressing *esgGal4^{ts}*, su(H)-Gal80>UAS-GFP were raised to adults

at 18°C (Gal4 is 'off') and then shifted to 29°C for 2 days. After feeding with sucrose, *P.e* bacteria or Dss, their intestines were dissected out for immunostaining with anti-GFP, PH3 and Pros (the EE cell marker) antibodies. We found PH3⁺ ISC with GFP in all three conditions (Fig. 2D-F'', white arrows). However, PH3⁺ expression was found in EBs (marked by GFP⁺Pros⁻ and small nuclei) only upon *P.e* infection (Fig 2E-E'', red arrows, G), but not upon sucrose and Dss feeding.

2) Moreover, the authors report the existence of EB-mitotic cells but do not really characterize them except for reporting that they are DI-. How different are they from regular EBs? Do they express Sox21a? Is *piezo* irregularly expressed in them? Do they have higher *esg* expression and therefore higher stemness potential? Or higher Notch expression than regular EBs? Also, how close are they to the basal membrane? Do they express more integrins? And therefore, could more integrins combined with closeness to the basal membrane influence them to divide? Since this study is about the identification of a functionally different subpopulation of EBs, the authors should characterize these cells by providing a set of additional markers to distinguish mitotic-EBs from non-mitotic EBs.

As suggested by the reviewer, we examined several markers, including *esg*, E(spl)mb-CD2 for Notch signaling, integrin and *piezo*. We found that *esg* was down-regulated in dividing EBs compared with non-dividing EBs (Fig EV5A-A''); however, others (E(spl)mb-CD2, integrin) remained unchanged (Fig EV5B-B', C-D'). The *piezo* expression is marked by *piezo*-GFP (GFP fused with *piezo* with the endogenous promoter), but it is too low to be detected. The expression of *piezo* was not up-regulated in dividing EBs. These results suggest that dividing EBs did not gain ISC fate. We mentioned these findings in the discussion (line 273-279).



The expression of *piezo* with *piezo*-GFP in the dividing EBs (red arrows).

3) The authors propose that this is a Ras/EGFR-dependent process. However, the authors do not test any other proliferative pathway. How specific is EB-mitosis to Ras/EGFR signaling? Can reduction of Hippo or induction JNK or JAK/STAT or Wnt also induce EB mitotic divisions. This is an important point to address that will be of interest to the whole intestinal regenerative field in *Drosophila*.

As suggested by the reviewer, we examined other signaling pathways, such as Wnt, Hh, Hippo and JNK. We found that activation of Wnt, Hh and JNK could not induce EB mitosis, and loss of *wts* could stimulate EB mitosis (Fig. EV7). We added it to the discussion (line 311-315).

4) The authors use the centromere UAS-Cnn-GFP marker or reduce mitosis with *stg*-RNAi under Su(H)>Ras induction. Since the premise of this paper is that EB-mitosis is a physiological phenomenon, the authors should use those tools during infection.

As suggested by the reviewer, we examined Cnn-GFP marker in dividing EBs upon *P.e* infection and added them to new Fig 5 (Fig 5L-N). We have examined EB mitosis when *stg* was knocked down in EBs upon *P.e* infection, and we found that blocking *stg* expression can reduce EB mitosis upon *P.e* infection (Fig EV2).

5) Finally, how dependent is EB-mitosis to ISC-mitosis? Is EBs-mitosis a compensatory mechanism for ISC proliferation? For example, if the authors after inducing infection (after proliferation has started) prevent temporally ISCs from dividing (e.g DITS> *stg*RNAi+ destabilizedGFP) and assay for mitotic-EBs (GFP-PH3+) how will those EBs behave? Will the levels of EB-mitosis decrease because ISC division is reduced? or will they increase and therefore act as a compensatory mechanism?

As suggested by the reviewer, we performed the experiment to prevent ISC proliferation by blocking *stg* expression with *DI-Gal4ts>UAS-stg-RNAi* and examined number of cells with EB mitosis upon *P.e* infection. We found that expression of *stg* in ISCs can significantly reduce overall PH3⁺ cell number (Fig 3A). After we quantified mitotic EBs (PH3⁺ GFP⁻Pros⁻) in intestines without or with *stg* expression in ISCs upon *P.e* infection, we found a slight reduction (Fig 3B), but no increase, indicating that EB mitosis does not act as a compensatory mechanism for ISC mitosis upon injury.

Reviewer 2

The experimental design is compelling, the experiments appear well done, and their results are interpreted rigorously. The text is well written and the figures properly presented. I believe that this work provides a significant shift in our understanding of the ISC lineage and should interest many colleagues in the field.

We thank the reviewer for these positive comments.

1) Although the experimental plan is pretty complete, the main conclusion of the paper relies on using a single Gal4-driven line (Su(H)-Gal4), as well as a single marker of ISCs (Delta). For example, Delta is also expressed in pre-EEs. Additional markers and ideally driver(s) would ascertain the taking home message of the study.

We performed an experiment with *esgGal4*, *su(H)-Gal80>UAS-GFP* to mark ISCs. In this experiment, the female flies expressing *esgGal4^{ts}*, *su(H)-Gal80>UAS-GFP* were raised to adults at 18°C (Gal4 is 'off') and then shifted to 29°C for 2 days. After feeding with sucrose, *P.e* bacteria or Dss, their intestines were dissected out for immunostaining with anti-GFP, PH3 and Pros (the EE cell marker) antibodies. We found PH3⁺ ISCs with GFP in all three conditions (Fig. 2D-F", white arrows). However, PH3⁺ expression was found in EBs (marked by GFP⁻Pros⁻ and small nuclei) only upon *P.e* infection (Fig 2E-E", red arrows, G), but not upon sucrose and Dss feeding.

2) Recent work has shown that the other type of ISC-derived progenitors, called pre-EEs and that will differentiate into Enteroendocrine cells (EEs), are also able of one round of division (e.g., Chen et al., Nat Cell Biol 2018). I guess it would be fair to introduce this notion, including in Fig 1A.

We added "divide" from pre-EE to EE cells in Fig 1A.

Minor points

Line 45-46: Work from the Perrimon lab using single cell RNA-seq (Hung et al., PNAS 2020) has provided evidence of the unsuspected diversity of *Drosophila* ECs and EEs, and revealed some populations exhibiting molecular signatures similar to Paneth cells, and to tuft and goblet cells, respectively.

We have rephrased this sentence (line 45).

Line 108: I guess that dividing EBs should be Ph3+ and GFP+?

We thank the reviewer for this suggestion and revised it (line 108).

Line 139: The non-cell-autonomous effects seen upon overexpression of EGFR ligands in EBs makes good sense. In contrast, how the activation of the EGF-RECEPTOR in EBs can lead to a similar phenotype is less clear. The authors mentioned in the discussion that EGF-r activation might be relayed by the JAK-STAT pathway. Have they evidence for that in EBs?

We examined the ligand expression of the JAK-STAT pathway when EGFR signaling was activated in EBs and found that *upd3* was up-regulated. Now we added this result to Fig EV6 (line 308).

Line 235: If I'm right, pre-EEs are also Prospero-positive. Do the authors have additional evidence for EB progeny can produce terminally differentiated EEs? If not, the pre-EE fate should be mentioned as well.

As suggested, we added the pre-EE fate in the revised text (line 248, 252).

Dear Dr Aiguo Tian,

Thank you for submitting your revised manuscript (EMBOJ-2022-110834R) to The EMBO Journal, as well as for your patience with our feedback, which got protracted by delayed reviewer input. Your amended study was sent back to the referees for their re-evaluation, and we have received comments from one of them, which I enclose below. Please note that while referee #2 was at this time not able to reassess the revised work, we have editorially evaluated your response to his/her earlier concerns and found them to be satisfactorily addressed. As you will see, the other expert stated that the work has been substantially improved by the complementary experiments and s/he is now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

We now need you to take care of a number of minor issues related to formatting and data presentation as detailed below, which should be addressed at re-submission.

Please contact me at any time if you have additional questions related to below points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

with
Best regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Please limit the keywords to the manuscript to maximally five.

>> Remove the author contributions information from the manuscript text. Note that CRediT has replaced the traditional author contributions section as of now because it offers a systematic machine-readable author contributions format that allows for more effective research assessment. and use the free text boxes beneath each contributing author's name to add specific details on the author's contribution. More information is available in our guide to authors.

<https://www.embopress.org/page/journal/14602075/authorguide>

>> Adjust the title of the 'Declaration of Competing Interests' section to 'Disclosure and Competing Interests Statement'.

>> Please add a separate 'Data availability section' to the Material and Methods stating 'No data amenable to database repository deposition were generated in this study.' .

>> Add a separate 'Statistical Analysis' section to the Material and Methods detailing the algorithms applied.

>> Callouts: please recheck callouts for Figures 1H; 4D,E,N,O; 7A ; 9A ;

>> Limit the number of EV figures to maximally five by either merging EV figures or adding supernumerary figures to an Appendix file as 'Appendix Figure S1, S2..', as one appendix PDF with a title page including a ToC, and Appendix Figure legends. Note that the callouts in the main text and legends need to be adjusted.

>> Please consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.

Further information is available in our Guide For Authors: <https://www.embopress.org/page/journal/14602075/authorguide>

Please use the link below to submit your revision:

Link Not Available

Referee #1:

Tian et al al, have a compelling story about how EBs undergo mitosis during infection. They find the EGFR-Ras pathway to be a major regulator of this process and leave room for potential involvement of the Hippo pathway in their discussion. In addition, they have further characterized these EB-mitotic cells as we requested. Overall, they have designed and explained their experiments properly, have sufficiently answered our questions and performed the experiments we requested. We recommend this paper for publication.

The authors performed the requested editorial changes.

Dear Dr Aiguo Tian,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper.

Also, in case you might NOT want the transparent process file published at all, you will also need to inform us via email immediately. More information is available here:

<https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess>

Please note that in order to be able to start the production process, our publisher will need and contact you shortly regarding the page charge authorisation and licence to publish forms.

Authors of accepted peer-reviewed original research articles may choose to pay a fee in order for their published article to be made freely accessible to all online immediately upon publication. The EMBO Open fee is fixed at \$6,100 USD / £4,950 GBP / €5,500 EUR (+ VAT where applicable).

We offer two licenses for Open Access papers, CC-BY and CC-BY-NC-ND.

For more information on these licenses, please visit:

http://creativecommons.org/licenses/by-nc-nd/3.0/deed.en_US

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

On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author(s) of the study. Please see the following link for representative examples and their integration into the article web page:

https://www.embopress.org/video_synopses

<https://www.embopress.org/doi/full/10.15252/emboj.2019103932>

Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

Finally, we have noted that the submitted version of your article is also posted on the preprint platform bioRxiv. We would appreciate if you could alert bioRxiv on the acceptance of this manuscript at The EMBO Journal in order to allow for an update of the entry status. Thank you in advance!

If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you for this contribution to The EMBO Journal and congratulations on a successful publication!

Please consider us again in the future for your most exciting work.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal
EMBO
Postfach 1022-40
Meyerhofstrasse 1
D-69117 Heidelberg
contact@embojournal.org
Submit at: <http://emboj.msubmit.net>

t

EMBO Press Author Checklist

Corresponding Author Name: Aiguo Tian
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2022-110834R

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](https://doi.org/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 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?	Not Applicable	
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	Material 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.	Yes	Material and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Yes	Material and Methods
Please detail housing and husbandry conditions .	Yes	Material and Methods
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	Material 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?	Not Applicable	
If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.		
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 .	Not Applicable	

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	