

Cell fragmentation in mouse preimplantation embryos induced by ectopic activation of the polar body extrusion pathway

Diane Pelzer, Ludmilla de Plater, Peta Bradbury, Adrien Eichmuller, Anne Bourdais, Guillaume Halet, and Jean-Léon Maitre **10.15252/emboj.2023114415**

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Transaction Report: This manuscript was transferred to The EMBO JOURNAL following peer review at Review Commons.

Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

The authors show that proximity of early mouse embryo blastomere chromosomes to the cell cortex activates the Polar Body Extrusion pathway to generate cell fragments. The authors use live cell imaging in control and Myo1C and dynein knockdown embryos to document accumulation of actin and myosin near chromosomes that come in close proximity to the cell cortex, which correlates with the increased fragmentation of the mutant blastomeres. The live imaging data are nicely presented and the results are well quantified. I have two major comments, and some minor comments on clarity, for the authors consideration in revising the manuscript.

****Major comment:****

1. The authors imply that Myo1 and dynein knockdowns result in an increase in the number of cells where chromosomes come in close proximity to the cell cortex. Apparently the spindle anchoring defects are meant to indicate that such defects are responsible for the increased frequency of abnormal chromosome proximity to the cortex. But the authors never actually document whether chromosomes in fact do come into proximity to the cortex more often in the mutant than in control embryos. The authors should clarify if they think the spindle anchoring defect does result in abnormal chromosome distributions. Can the authors somehow quantify a defect in overall chromosome positioning in mutant vs control blastomeres? Presumably the movies the authors already have could be used to provide such quantification?
2. Near the end of the paper, the authors discuss how cell with bent/un-anchored spindles are more prone to fragmentation, referring to Figure 2. But Figure 2 does not document a correlation between blastomeres with bent spindles and increased fragmentation. Rather it shows an increase in bent spindles and in fragmentation in mutant vs control, but does show that they occur together. The authors should more accurately describe their results or provide such a correlation with additional data.
3. Finally, in describing the data in Figure 3, the authors refer to persistence of the spindle and bending of the spindle as indicating problems with anchoring. It is not clear to me how either spindle persistence or bending relate to anchoring. The authors should explain how they are related if they are, and it would be better if the authors could document spindle displacement relative to the cell center or cortex to make their point more directly that anchoring is defective.

****Minor comment.****

The authors document in Figure 3 that Myo1C KO blastomeres have an enhanced response, with more myosin accumulating at the cortex in response to chromosomes. Why does knocking out one non-muscle myosin lead to enhanced accumulation of another? The authors note this effect but provide no discussion as to how it occurs. Some clarification might be helpful.

2. Significance:

Significance (Required)

The authors provide a significant advance in our understanding of why early mammalian embryos, especially early human embryos, are so prone to fragmentation. Their data strongly support their conclusion that increased proximity of chromosomes to the cortex does lead to activation of the PBE response, which is an interesting and well documented finding. However, unless the authors can address my major comments and provide more direct evidence for increased displacement of chromosomes being responsible for increased fragmentation, they should revise their manuscript to acknowledge that they have not directly quantified chromosome positioning and thus do not conclusively document that it is responsible for increased fragmentation in the mutant oocytes.

3. How much time do you estimate the authors will need to complete the suggested revisions:

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Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

The manuscript "Ectopic activation of the polar body extrusion pathway triggers cell fragmentation in preimplantation embryos" by Pelzer and colleagues is focused on mechanism of cell fragmentation in early preimplantation embryos. This is an important issue, since fragmentation, with subsequent cell loss, has significant impact on early development of human embryos in vitro.

To study the cell fragmentation within the embryo, authors used mouse model system. However, since during the mouse preimplantation development blastomere fragmentation is less frequent than in human embryos, they used knockout of unconventional myosin-Ic to induce fragmentation of embryonic blastomeres with higher frequency and a similar morphology, known from human embryos.

Using their Myo1c KO, authors confirmed previous observation that reduction of myosin-Ic impairs spindle anchoring and they further show that the defects in spindle anchoring are linked to cell fragmentation. And that similar defects could be induced by chemical inhibition of dynein. Importantly, the defects in anchoring, causing aberrant spindle movements, bring spindle and chromosomal DNA to the proximity of the cell cortex. This induces local changes in concentration and organization of actin and myosin IIA and leads into fragmentation. Authors show that this pathway shares similarity with mechanism of polar body extrusion (PBE) during meiosis, namely that it requires active Cdc42-mediated actin polymerization or Ect2 signaling. And also, that important role in cell fragmentation is played by cell surface tension. Based on their results, authors propose that cell fragmentation within the embryo is triggered either by hyperactivation of PBE pathway in cells with normal surface tension, or by PBE pathway activation in cells with higher contractility.

This manuscript brings important information about mechanism, which might contribute to the high incidence of blastomere fragmentation in human embryos. I

have not identified any important issues with experimental work or conclusions and therefore I recommend this paper for publication. The results from the mouse model system however need to be verified by further studies in human or similar embryos, which naturally exhibit higher fragmentation.

2. Significance:

Significance (Required)

This study revealed important mechanism, which might be responsible for inducing fragmentation of blastomeres in early preimplantation embryos. Authors use mouse knockout model system and therefore the results should be verified in other species, in which the embryos show higher fragmentation naturally. The manuscript provides evidence that pathway, leading into PBE in oocytes, remains operational also in embryos and might contribute to blastomere fragmentation in case when spindle loses anchoring to the membrane. The results of this manuscript should be of interest not only to the researchers in reproduction, but also to the general audience.

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Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

The manuscript discussed interesting and relevant topics in which the Authors addressed the effects of mouse Myo1C knock out on cell fragmentation and spindle anchoring defects. The authors found that fragmentation occurs in mitosis after ectopic activation of actomyosin contractility by signals emanating from DNA.

2. Significance:

Significance (Required)

This is an excellent report dealing with significant technical methodologies. I find no fault in the methods, data analysis, or conclusions. I only have two comments. First, the authors should expand on the previous findings about the role of Myo1c during early preimplantation development. Second, the discussion should be expanded to compare the results of this study with those of previous/related studies (e.g., other factors involve in fragmentation and spindle anchoring). Finally, I was not able to open movie#2 and movie#8 so they may need to be re-uploaded.

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Estimated time to Complete Revisions (Required)

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We thank all reviewers for their careful analysis of our manuscript, constructive suggestions and support of our work.

Reviewer 1

The authors show that proximity of early mouse embryo blastomere chromosomes to the cell cortex activates the Polar Body Extrusion pathway to generate cell fragments. The authors use live cell imaging in control and Myo1C and dynein knockdown embryos to document accumulation of actin and myosin near chromosomes that come in close proximity to the cell cortex, which correlates with the increased fragmentation of the mutant blastomeres. The live imaging data are nicely presented and the results are well quantified. I have two major comments, and some minor comments on clarity, for the authors consideration in revising the manuscript.

Major comment:

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We thank the reviewer for this opportunity to correct our previous assumptions. Following the reviewer's suggestion, we tracked the distance between the cell surface and the center of the chromosomes cluster throughout mitosis. We found little difference in this distance between control and Myo1cKO embryos (Fig S3a), unlike what we had initially implied. This distance seemed more variable in Myo1cKO embryos than in control ones, suggesting that chromosome movements may be more erratic but analysis of this variation for individual cells did not show consistent differences between control and Myo1cKO embryos either (Fig S3b). Therefore, we cannot explain the increased signaling with differences in proximity of the chromosomes to the cortex during mitosis.

Instead, as already hinted in our initial manuscript as an additional factor, we find that signaling from chromosomes to the cortex can occur for an extended time in embryos with impaired spindle anchoring.

We had already measured that mitotic spindles persisted for a longer time in Myo1cKO embryos than in control ones (Fig 2b), as well as in ciliobrevin treated embryos as compared to DMSO treated ones (Fig S2b). To strengthen this data, we performed additional experiments in which we injected mRNA encoding fluorescent lamin-associated protein 2b (Lap2b-GFP) to track the breakdown and reassembly of the nuclear envelope. Consistent with the mitotic spindle persisting for a longer time in Myo1cKO embryos than in control ones, it generally takes more time for Myo1cKO embryos to reassemble their nuclear envelope than for control embryos (50 min vs 70 min, $n = 8$ control and 15 Myo1cKO embryos, $p = 0.0161$, Fig S3c-d, Movie 5). Taken together, the nuclear envelope and spindle data indicate that, although chromosomes are not closer to the cortex in Myo1cKO embryos than in control ones, they spend more time outside of the nucleus. This should give chromosomes extended opportunities to signal to the cortex and explains how difficulties with chromosome separation can lead to the hyper-activation of the polar body extrusion pathway.

We have revised our manuscript accordingly.

2. Near the end of the paper, the authors discuss how cell with bent/un-anchored spindles are

more prone to fragmentation, referring to Figure 2. But Figure 2 does not document a correlation between blastomeres with bent spindles and increased fragmentation. Rather it shows an increase in bent spindles and in fragmentation in mutant vs control, but does show that they occur together. The authors should more accurately describe their results or provide such a correlation with additional data.

We thank the referee for pointing out this missing information.

To support our conclusions, we now provide additional analyses of mitosis duration in non-fragmenting and fragmenting cells from Myo1cKO embryos. When cells fragment, their mitosis is consistently longer, as measured from the persistence of the mitotic spindle, than when not fragmenting (Fig 2c). This provides a direct correlation between spindle defects and fragmentation.

We now present these analyses in the revised manuscript.

3. Finally, in describing the data in Figure 3, the authors refer to persistence of the spindle and bending of the spindle as indicating problems with anchoring. It is not clear to me how either spindle persistence or bending relate to anchoring. The authors should explain how they are related if they are, and it would be better if the authors could document spindle displacement relative to the cell center or cortex to make their point more directly that anchoring is defective.

We apologize for not making this clearer in our initial manuscript. As others noted before (Kotak et al, 2012; Mangon et al, 2021), poorly anchored spindles show larger displacements or rotations during mitosis. Spindle persistence and bending may not be directly related to spindle anchoring defects but could reflect broader issues with spindle assembly and function caused by spindle anchoring defects. Since a previous in vitro study had identified that Myo1cKO is important for spindle anchoring (Mangon et al 2021) and that ciliobrevin, known for compromising spindle anchoring, phenocopied these aspects, we had initially focused on anchoring defects in our conclusions. We still stand by our conclusion that our data suggest spindle anchoring defects. Nevertheless, we agree that our observations report more general spindle defects and that anchoring may be only one of the defective aspects. Instead of “*spindle anchoring defects*”, we now simply mention “*spindle defects*” unless specifically discussing spindle straightness and rotation.

Minor comment.

The authors document in Figure 3 that Myo1C KO blastomeres have an enhanced response, with more myosin accumulating at the cortex in response to chromosomes. Why does knocking out one non-muscle myosin lead to enhanced accumulation of another? The authors note this effect but provide no discussion as to how it occurs. Some clarification might be helpful.

In our manuscript, we report that chromosome proximity to the cortex is associated with Cdc42 activation, which leads to cortical actin recruitment (Fig 4a-d). We also observe that non-muscle myosin II (Myh9) is recruited to the cortex when chromosomes come near (Fig 3d-f). Importantly, these phenomena occur in control embryos as well and not only in Myo1cKO embryos.

We propose that this recruitment is further increased in Myo1cKO embryos (Fig 3f) because chromosomes spend more time outside of the nuclear envelope (Fig 2). This leads to fragmentation and is not specific to Myo1cKO since the same occurs after ciliobrevin treatment (Fig S2).

The authors provide a significant advance in our understanding of why early mammalian embryos, especially early human embryos, are so prone to fragmentation. Their data strongly support their conclusion that increased proximity of chromosomes to the cortex does lead to activation of the PBE response, which is an interesting and well documented finding. However, unless the authors can address my major comments and provide more direct evidence for increased displacement of chromosomes being responsible for increased fragmentation, they should revise their manuscript to acknowledge that they have not directly quantified chromosome

positioning and thus do not conclusively document that it is responsible for increased fragmentation in the mutant oocytes.

We thank the reviewer for their thorough analysis of our data and for giving us the opportunity to correct some of the aspects of our study.

Reviewer 2

The manuscript "Ectopic activation of the polar body extrusion pathway triggers cell fragmentation in preimplantation embryos" by Pelzer and colleagues is focused on mechanism of cell fragmentation in early preimplantation embryos. This is an important issue, since fragmentation, with subsequent cell loss, has significant impact on early development of human embryos in vitro.

To study the cell fragmentation within the embryo, authors used mouse model system. However, since during the mouse preimplantation development blastomere fragmentation is less frequent than in human embryos, they used knockout of unconventional myosin-Ic to induce fragmentation of embryonic blastomeres with higher frequency and a similar morphology, known from human embryos.

Using their Myo1c KO, authors confirmed previous observation that reduction of myosin-Ic impairs spindle anchoring and they further show that the defects in spindle anchoring are linked to cell fragmentation. And that similar defects could be induced by chemical inhibition of dynein. Importantly, the defects in anchoring, causing aberrant spindle movements, bring spindle and chromosomal DNA to the proximity of the cell cortex. This induces local changes in concentration and organization of actin and myosin IIA and leads into fragmentation. Authors show that this pathway shares similarity with mechanism of polar body extrusion (PBE) during meiosis, namely that it requires active Cdc42-mediated actin polymerization or Ect2 signaling. And also, that important role in cell fragmentation is played by cell surface tension. Based on their results, authors propose that cell fragmentation within the embryo is triggered either by hyperactivation of PBE pathway in cells with normal surface tension, or by PBE pathway activation in cells with higher contractility.

This manuscript brings important information about mechanism, which might contribute to the high incidence of blastomere fragmentation in human embryos. I have not identified any important issues with experimental work or conclusions and therefore I recommend this paper for publication. The results from the mouse model system however need to be verified by further studies in human or similar embryos, which naturally exhibit higher fragmentation.

We thank the reviewer for their careful examination of our manuscript and data.

We agree that it would be important to verify the validity of our findings in other species. We have considered performing experiments with human embryos.

Ideally, we would need embryos in their early cleavage stages (zygote to 4-cell stages) to be able study fragmentation without perturbing morphogenetic movements, which begin at the 8-cell stage. Such early embryos are particularly rare, which further requires careful experimental design.

Ideally, such carefully designed experiment would not cause additional fragmentation (as we have mostly done in the present study) but rather reduce this deleterious process. In light of our experiments shown in Fig 4c-d, inhibiting Cdc42 would be a good way to reduce polar body extrusion signaling. Injection of DNCdc42 mRNA would be embryo-consuming to setup. We tried a Cdc42 chemical inhibitor on mouse embryos with unreliable results. Therefore, we do not yet feel confident in using precious human embryos with our currently available options.

Another complication is administrative since this project was funded by the ERC, which does not allow experimentation with human embryos.

As for studying the phenomenon in species other than mouse or human, we currently have limited access to other mammalian species. Generally, other mammalian embryos are less well

characterized and, in particular, the species-specific fragmentation behavior would need to be characterized before initiating any attempt to reduce it.

We hope that the reviewers will agree that the current manuscript, describing and dissecting a previously unknown mechanism, makes sufficient advances to be published without the need to assess its evolutionary conservation.

This study revealed important mechanism, which might be responsible for inducing fragmentation of blastomeres in early preimplantation embryos. Authors use mouse knockout model system and therefore the results should be verified in other species, in which the embryos show higher fragmentation naturally. The manuscript provides evidence that pathway, leading into PBE in oocytes, remains operational also in embryos and might contribute to blastomere fragmentation in case when spindle loses anchoring to the membrane. The results of this manuscript should be of interest not only to the researchers in reproduction, but also to the general audience.

Reviewer 3

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

The manuscript discussed interesting and relevant topics in which the Authors addressed the effects of mouse Myo1C knock out on cell fragmentation and spindle anchoring defects. The authors found that fragmentation occurs in mitosis after ectopic activation of actomyosin contractility by signals emanating from DNA.

Reviewer #3 (Significance (Required)):

This is an excellent report dealing with significant technical methodologies. I find no fault in the methods, data analysis, or conclusions. I only have two comments. First, the authors should expand on the previous findings about the role of Myo1c during early preimplantation development. Second, the discussion should be expanded to compare the results of this study with those of previous/related studies (e.g., other factors involve in fragmentation and spindle anchoring). Finally, I was not able to open movie#2 and movie#8 so they may need to be re-uploaded.

We thank the reviewer for their careful assessment of our study.

We apologize for not discussing enough the previous research on Myo1c. To our knowledge, there is only one previous study reporting the effect of a point mutation on Myo1c on mouse ear physiology (Stauffer et al 2005). This is the first study on the role of Myo1c during mouse development. At this point, we would like to stress that our study, while partially based on the KO of Myo1c, is about cell fragmentation, which we induce experimentally in three independent ways: Myo1c KO, ciliobrevin treatment or Ect2 overexpression.

Regarding fragmentation, to our knowledge there is simply no convincing mechanism to explain this phenomenon. One study proposed that membrane threads connecting the cell surface to the zona pellucida could pull on cells and promote fragmentation (Derick et al 2017). However, fragmentation also occurs without zona pellucida, and hence without threads pulling on cells' surfaces (Yumoto et al 2020). Other than that, fragmentation was associated with mitosis and general cytoskeleton defects, without no clear mechanism (Alikani 1999, Fujimoto et al 2011, Daughtry et al 2019).

We have now expanded these discussions.

Dear Dr. Maitre,

Thank you for submitting your revised Review Commons manuscript to The EMBO Journal. Your manuscript has now been seen by one of the original reviewers, who finds that their main concerns have been addressed and now recommends publication of the manuscript. I will therefore be happy to accept the manuscript for publication in The EMBO Journal after its reformatting as indicated below.

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Please feel free to contact me if you have any further questions regarding this final editorial revision. Please use the link below to upload the revised files.

Thank you for the opportunity to consider your work for publication, and I look forward to receiving your revised manuscript.

With best regards,

Ieva

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Senior Scientific Editor
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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 (28th Aug 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #1:

Pelzer et al describe their results documenting that delays in mitosis in pre-implantation mouse embryos result in oocyte fragmentation, which the authors propose is due to the chromosomes being exposed to the cytoplasm for longer and thus able to productively signal through the polar body extrusion pathway to cause extrusion like events that lead to oocyte fragmentation. The authors have substantially improved the manuscript since its original submission by documenting that it is a cell cycle delay, not chromosome proximity to the membrane, that results in ectopic activation of the polar body extrusion pathway and abnormal oocyte fragmentation. These results provide important new insights into this process and in my opinion warrants publication without any further revision.

The authors have addressed all editorial requests.

Dear Dr. Maitre,

Thank you for addressing the final editorial points. I sincerely apologise for the delay in communicating the decision due to the high number of submissions we receive at the moment. I am now pleased to inform you that your manuscript has been accepted for publication.

Before we forward your manuscript to our publishers, I would like to propose a couple of minor changes in the article title, synopsis and abstract to enhance their accessibility to our more general readership. I have also written a short blurb that will accompany the title of your manuscript in our online table of contents. Please take a look at the text below and in the attached manuscript text file and let me know if any corrections are necessary.

Title:
Cell fragmentation in mouse preimplantation embryos induced by ectopic activation of the polar body extrusion pathway

Blurb:
Proximity of chromosomes to the cell cortex induces excessive contractions of the actomyosin cortex and cell disintegration in the early mouse embryo.

Synopsis:
Cell fragmentation is a poorly understood process and a major defect commonly observed in human embryos. Here, a study in mouse preimplantation embryos reveals that ectopic activation of a meiotic pathway during mitosis triggers cell fragmentation via induction of a local actomyosin contraction.

- Inhibition of the motor proteins Myo1c or dynein leads to cell fragmentation in mouse embryos.
- Ectopic contractions of the embryonic actomyosin cortex pinch off cell fragments.
- In contrast to other mitotic cells where chromosomes induce polar relaxation, the proximity of DNA to the cortex promotes contraction in mouse embryo cells.
- Ectopic contractions are triggered by the continuous post-meiotic activation of the polar body extrusion pathway components Cdc42 and Ect2.

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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!

Best regards,

Ieva

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Title: Fragmentation of preimplantation embryos by ectopic activation of the polar body extrusion pathway

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- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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- 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:
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 - 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	NA
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.	Yes	Materials and Methods
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	NA
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	NA
Report if the cell lines were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	NA
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	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	NA
Please detail housing and husbandry conditions .	Yes	Materials and Methods
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Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	NA
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	NA
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?	Yes	Acknowledgements
Design	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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	NA
Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	NA

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	NA

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	Materials and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, have they been described?	Yes	Materials and Methods
Include a statement about blinding even if no blinding was done.	Yes	Materials and Methods
Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Yes	No sample was excluded
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?	Yes	Materials and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was replicated in laboratory.	Yes	Legends
In the figure legends: define whether data describe technical or biological replicates .	Yes	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	NA
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	NA
Studies involving human participants : For publication of patient photos , include a statement confirming that consent to publish was obtained.	Not Applicable	NA
Studies involving experimental animals : State details of authority granting ethics approval (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
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	NA

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	no
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	no
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	NA

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	NA
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	NA
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	NA

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have primary datasets been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Materials and Methods
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	NA
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	NA
If publicly available data were reused, provide the respective data citations in the reference list .	Yes	Materials and Methods