EMBO Molecular Medicine

Development of a novel therapy for systolic heart failure

Hamsa Puthalakath, Corey Pollock, Xilun Wang, Hussam Alsaraji, Joseph Menassa, George Mbogo, Dimuthu Angage, Benjamin Richards, Jason Glab, Keshava Datta, Liana Theodoridis, Steve Petrovski, Daniel Donner, Yuvixza Lizarme-Salas, Xiao-Jun Du, Michael Foley, Brian Smith, and Belinda Abbott

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Editor: Zeljko Durdevic

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.)

22nd Jan 2025

Dear Dr. Puthalakath,

Thank you for the submission of your manuscript to EMBO Molecular Medicine, and please accept my apologies for the unusual delay in getting back to you. We have now received feedback from two of the three reviewers who agreed to evaluate your manuscript. As the referee #3 will unfortunately not be able to return his/her report in a timely manner, and given that both reviewers provide similar recommendations, we prefer to make a decision now in order to avoid further delay in the process. Should referee #3 provide a report, we will send it to you, with the understanding that we will not ask for an additional revision.

As you will see from their reports pasted below, both referees recognize potential interest of the manuscript but also raise important concerns that should be addressed in a major revision. Confirmation of the main findings in an additional animal model as suggested by the referee #2 should be the focus of the revision. Given that the revision will require extensive experimentation we think six months rather than three months would be more appropriate to provide the complete revision. If you would like to discuss further the points raised by the referees, I am available to do so via email or video. Let me know if you are interested in this option.

Further consideration of a revision that addresses reviewers' concerns in full will entail a second round of review. EMBO Molecular Medicine encourages a single round of revision only and therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. For this reason, and to save you from any frustrations in the end, I would strongly advise against returning an incomplete revision.

We would welcome the submission of a revised version within six months for further consideration. Please let us know if you require longer to complete the revision.

I look forward to receiving your revised manuscript.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Editor
EMBO Molecular Medicine

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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). For guidance, download the 'Figure Guide PDF': (https://www.embopress.org/page/journal/17574684/authorguide#figureformat).

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In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.

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10) 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.

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Please also suggest a striking image or visual abstract to illustrate your article as a PNG file 550 px wide x 300-600 px high.

15) Include a Reagents and Tools Table as part of the Methods section, which can be downloaded from our author guidelines (https://www.embopress.org/page/journal/17574684/authorguide#structuredmethods)

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

This paper presents a novel hemodynamically neutral therapy for heart failure. By establishing cell models, conducting high-throughput screening, and refining the structure-activity relationship, the lead compound BR43 was identified. Through a series of validations, including in vitro experiments, animal models, and target identification, BR43 was proposed as a promising candidate for treating heart failure, offering potential improvements over current clinical therapies. This work demonstrates strong innovation and potential, with significant practical value for heart failure treatment.
However, the paper still has some shortcomings: 1. Although the authors suggest that the entire signaling pathway is related to apoptosis, specific cellular and biochemical phenotypes should be provided to clearly demonstrate how the drug induces apoptosis; 2. In the cell proliferation experiments, it would be helpful to include more concentration points, plot dose-response curves, and calculate the IC50 values. This would allow for a more direct comparison of the drug's safety profile. Additionally, it should be clarified whether the observed phenotype is indeed due to apoptosis. 3. In the animal models, there is a lack of evaluation using relevant biomarkers of heart failure, such as BNP, which would help to clearly demonstrate the effect of BR43 on the disease improvement. 4. It would be beneficial if the authors could present more detailed results from mass spectrometry and provide a more thorough description of the process used to screen candidate proteins. 5. Given the challenges in performing affinity experiments for Wdr3, it would be helpful for the authors to use additional computational methods to predict the binding affinity between BR43 and Wdr3. 6. It is noted that there are few references from the past five years in the manuscript. The authors are encouraged to add more recent references to ensure the paper is up-to-date.
This paper provides a comprehensive account of the discovery and modification of a compound, along with in vitro and in vivo experiments, proposing BR43 as a candidate for treating heart failure. The experimental results suggest that BR43 has promising clinical application potential and offers valuable theoretical and practical guidance for drug development in heart failure.

Referee #2 (Comments on Novelty/Model System for Author):

This is a novel study, aiming at identifying new compounds to treat heart failure. The authors have employed a number of techniques and convincingly demonstrated BR43 as a candidate drug to treat heart failure.
The reasons of my less enthusiastic recommendation are the follows.
1-The in vivo model is isoproterenol injection. Whereas it is appropriate, it should not be the only one. I would recommend additional mouse models, including genetic models to be tested.
2-Data quality. Some immunoblots should be improved and quantified.

Referee #2 (Remarks for Author):

In this study, Pollock et al. focused on the identification of novel compounds to treat heart failure. The authors started with screening for similar or better inhibitors of beta-AR signaling and cell death in H9c2 cells. They also investigated the effects on

PKA signaling and Bim expression. They next spent many efforts to narrow down and refine the candidate compounds. In the end, they generated a modified version of their leading compounds. In vivo tests showed the this BR43 compound significantly improved isoproterenol-induced heart failure and also inhibited the expression of Bim.

1. The authors started with beta-AR signaling as the foundation for screening. However, later, they more focused on beta-AR-independent signaling, which is likely better considering the plethoric effect of inhibiting beta-AR in vivo. This is interesting. However, the in vivo test still used isoproterenol in mice. What about the therapeutic effects of BR43 in conventional heart failure model, such as transverse aortic constriction?
2. Along the similar line as in (1), it would be better if the authors could conduct a side-by-side comparison to delineate the effects of BR43 as opposed to other mainstream drugs in vivo.
3. Some immunoblots are suboptimal. In addition, quantification should be conducted for all immunoblots.
4. In addition to H9c2 cells, primary neonatal or adult myocytes should be employed. If possible, human iPSC-Cardiomyocytes may be used.

Dear Editors,

Thank you for reviewing our manuscript and thank you for the valuable and constructive feedback from the reviewers. We would also like to thank the editors for giving us enough time to conduct the additional animal experiments (obtaining ethics approval from the relevant bodies has its own challenges and we appreciate the editors taking this into consideration). We have addressed all the reviewers' comments as given below:

Reviewer 1:

1. Although the authors suggest that the entire signaling pathway is related to apoptosis, specific cellular and biochemical phenotypes should be provided to clearly demonstrate how the drug induces apoptosis.
   Answer: There must be some misunderstanding. The drug does not induce apoptosis. It blocks apoptosis by reducing b-AR-mediated Bim induction. How Bim induces apoptosis is well documented by us and various others (Puthalakath et al, Cell 129 (7), 1337-1349; Puthalakath et al, Molecular cell 3 (3), 287-296).

2. In the cell proliferation experiments, it would be helpful to include more concentration points, plot dose-response curves, and calculate the IC50 values. This would allow for a more direct comparison of the drug's safety profile. Additionally, it should be clarified whether the observed phenotype is indeed due to apoptosis.
   Answer: We have done IC50 measurements, and it is part of Figure 2C in the revised manuscript. Again, the observed phenotype is due to apoptosis inhibition rather than due to apoptosis.
3. 3. In the animal models, there is a lack of evaluation using relevant biomarkers of heart failure, such as BNP, which would help to clearly demonstrate the effect of BR43 on the disease improvement.
   Answer: In the revised manuscript, we have used an additional animal model, and we measured serum troponin levels (a marker for heart failure). It is part of Figure 3 i.e., 3D.
4. 4. It would be beneficial if the authors could present more detailed results from mass spectrometry and provide a more thorough description of the process used to screen candidate proteins.

   Answer: Very detailed description of the mass spectrometry analyses is given in the methods section under three subheadings: 1. *Sample preparation for proteomic analysis,* 2. *Mass spectrometry data acquisition* and 3. *Database searches and bioinformatics analysis*.

5. Given the challenges in performing affinity experiments for Wdr3, it would be helpful for the authors to use additional computational methods to predict the binding affinity between BR43 and Wdr3.

   Answer: The "Molecular modelling" section of the Results provides the results of our investigations into the binding affinity between BR43 and Wdr3; in short, the ligands were first docked with the receptor, then molecular dynamics simulations conducted prior to estimating the binding affinity. These are all described in the "Molecular modelling" section of the Methods section.

6. It is noted that there are few references from the past five years in the manuscript. The authors are encouraged to add more recent references to ensure the paper is up-to-date.
   Answer: Thanks for pointing this out. There is lack of research into developing hemodynamically neutral drugs for treating HFrEF. We had mentioned a few recent attempts such as Sacubitril/Valsatran and SGLT-2. However, we inadvertently omitted Omecamtiv Mecarbil (OM). Though it was touted to be a hemodynamically neutral therapy by activating myosin, FDA approval was denied due to lack of clinical efficacy in 2024. This has been added in the discussion in the revised manuscript.

Reviewer 2:

1. The authors started with beta-AR signaling as the foundation for screening. However, later, they more focused on beta-AR-independent signaling, which is likely better considering the plethoric effect of inhibiting beta-AR in vivo. This is interesting. However, the in vivo test still used isoproterenol in mice. What about the therapeutic effects of BR43 in conventional heart failure model, such as transverse aortic constriction?
   Answer: We have conducted additional animal experiments i.e., doxorubicin-induced cardiotoxicity model and this data is included in the revised manuscript (Figure 3D).
2. Along the similar line as in (1), it would be better if the authors could conduct a side-by-side comparison to delineate the effects of BR43 as opposed to other mainstream drugs in vivo.
   Answer: Indeed, we have compared BR43 side by side with a clinically used beta blocker atenolol and this data is included in the revised manuscript (Figure 3D). We also had compared BR43 with pindolol in Fig. 1C and Fig. 2D.
3. 3. Some immunoblots are suboptimal. In addition, quantification should be conducted for all immunoblots.
   Answer: Figure 3C was pixelated on magnification. This error has been fixed with a new panel with higher resolution. We have also quantified all the immunoblots using ImageJ but due to the crowded nature of the figure panels, we have submitted a separate spreadsheet with the values and figures.
4. 4. In addition to H9c2 cells, primary neonatal or adult myocytes should be employed. If possible, human iPSC-Cardiomyocytes may be used.
   Answer: Indeed, we did not rely only on H9C2 cells. Figure 3C is using mouse cardiac tissues (primary adult myocytes).

20th Jun 2025

Dear Dr. Puthalakath,

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1) Authors:
- E-mail correspondence to Keshavakrishna Datta could not be delivered. Please update the e-mail address and make sure to enter correct e-mail addresses for all authors in our submission system.
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3. Please note that information related to n is missing in the legend of figure EV5 C.
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5. Please note that the error bars are not defined in the legend of figure EV5 C.
- Rename "Competing interest" to "Disclosure and competing interests statement". We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary.
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- Indicate in legends exact n and exact p values, not a range, along with the statistical test used. To keep the figures "clear" some authors found providing an Appendix table Sx with all exact p-values preferable. You are welcome to do this if you want to.
- In Methods, provide the antibody dilutions that were used for each antibody.
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https://www.embopress.org/page/journal/17574684/authorguide#structuredmethods
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- Please check your synopsis text and image before submission with your revised manuscript. Please be aware that in the proof stage minor corrections only are allowed (e.g., typos).

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11) Please provide a point-by-point letter INCLUDING my comments as well as the reviewer's reports and your detailed responses (as Word file).

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Senior Editor
EMBO Molecular Medicine

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3) supplemental information as Expanded View and/or Appendix. Please carefully check the authors guidelines for formatting Expanded view and Appendix figures and tables at
https://www.embopress.org/page/journal/17574684/authorguide#expandedview

4) a letter INCLUDING the reviewer's reports and your detailed responses to their comments (as Word file).

5) The paper explained: EMBO Molecular Medicine articles are accompanied by a summary of the articles to emphasize the major findings in the paper and their medical implications for the non-specialist reader. Please provide a draft summary of your article highlighting
- the medical issue you are addressing,
- the results obtained and
- their clinical impact.
This may be edited to ensure that readers understand the significance and context of the research.
Please refer to any of our published articles for an example.

6) Author contributions: the contribution of every author must be detailed in a separate section.

7) EMBO Molecular Medicine now requires a complete author checklist (https://www.embopress.org/page/journal/17574684/authorguide) to be submitted with all revised manuscripts. Please use the checklist as guideline for the sort of information we need WITHIN the manuscript. The checklist should only be filled with page numbers were the information can be found. This is particularly important for animal reporting, antibody dilutions (missing) and exact values and n that should be indicted instead of a range.

8) Every published paper now includes a 'Synopsis' to further enhance discoverability. Synopses are displayed on the journal webpage and are freely accessible to all readers. They include a short stand first (maximum of 300 characters, including space) as well as 2-5 one sentence bullet points that summarise the paper. Please write the bullet points to summarise the key NEW findings. They should be designed to be complementary to the abstract - i.e. not repeat the same text. We encourage inclusion of key acronyms and quantitative information (maximum of 30 words / bullet point). Please use the passive voice. Please attach these in a separate file or send them by email, we will incorporate them accordingly.

You are also welcome to suggest a striking image or visual abstract to illustrate your article. If you do please provide a jpeg file 550 px-wide x 300-600px high.

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***** Reviewer's comments *****

Referee #1 (Remarks for Author):

The revised manuscript is suitable for publication.

The authors addressed the remaining editorial issues.

17th Jul 2025

Dear Dr. Puthalakath,

We are pleased to inform you that your manuscript is accepted for publication and is now being sent to our publisher to be included in the next available issue of EMBO Molecular Medicine.

Your manuscript will be processed for publication by EMBO Press. It will be copy edited and you will receive page proofs prior to publication. Please note that you will be contacted by Springer Nature Author Services to complete licensing and payment information.

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If you have any questions, please do not hesitate to contact the Editorial Office. Thank you for your contribution to EMBO Molecular Medicine.

Yours sincerely,
Zeljko Durdevic

Zeljko Durdevic
Senior Editor
EMBO Molecular Medicine

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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
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  - are tests one-sided or two-sided?
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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	Reagent and Tools Table

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	Reagent and Tools Table

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	Reagent and Tools Table

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Reagent and Tools Table
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**.	Yes	Materials and Methods section under the subsection: Animal experiments

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

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	The La Trobe University proteomics research platform mentioned in the acknowledgement section

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	In each figure legend, wherever applicable
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	In the animal experiment section of Materials and Methods
Include a statement about **blinding** even if no blinding was done.	Yes	In the animal experiment section of Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	

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	Under the figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Under the figure legends (n vs N)

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 approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Under Materials and Methods, subsection: Animal experiments
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

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

Reporting

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

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

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data availability statement section has been added
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?	Yes	Provided in the methods section and in the reagents and tools table
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	