EMBO Molecular Medicine

FLT1 activation in cancer cells promotes PARP-inhibitor resistance in breast cancer

Swarnali Acharyya, Anup Biswas, Yifan Tai, Angela Chow, Seoyoung Han, Courtney Coker, Wanchao Ma, Yifan Gu, Valeria Estrada Navarro, Manoj Kandpal, Hanina Hibshoosh, Kevin Kalinsky, Katia Manova-Todorova, Anton Safonov, Elaine Walsh, Mark Robson, Larry Norton, Richard Baer, and Taha Merghoub

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(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.)

5th Oct 2023

Dear Dr. Acharyya,

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now received feedback from the three reviewers who agreed to evaluate your manuscript. As you will see below, the reviewers raise substantial concerns on your work, which unfortunately preclude its publication in EMM in its current form.

The reviewers find that the question addressed by the study is of potential interest, however they remain unconvinced that some of the major conclusions are sufficiently supported by the data. They thus raise issues related (but not limited) to the reliance on loss-of-function experiments, lack of control/rescue experiment regarding Flt1 suppression, insufficient mechanistic insight, lack of comparison with other inhibitors, and unclear human data.

After further consultation with the referees, they agreed that the phospho-proteomic analyses and organoid experiments could be optional for revisions. Regarding the novelty, we would like you to clearly discuss the translational advance brought forward in your manuscript.

If you feel you can satisfactorily address the referees' points, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will once again be subject to review, and we cannot guarantee at this stage that the eventual outcome will be favorable.

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.

We are expecting your revised manuscript within three months, if you anticipate any delay, please contact us.

When submitting your revised manuscript, please carefully review the instructions that follow below. We perform an initial quality control of all revised manuscripts before re-review; failure to include requested items will delay the evaluation of your revision.

We require:

1) A .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) Individual production quality figure files as .eps, .tif, .jpg (one file per figure). For guidance, download the 'Figure Guide PDF' (https://www.embopress.org/page/journal/17574684/authorguide#figureformat).

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

4) A .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) A complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/17574684/authorguide#submissionofrevisions). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. An ORCID identifier is currently missing for Anup K Biswas.

7) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/17574684/authorguide#dataavailability).

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

8) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.). Please provide exact p values.

9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

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.
- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc.
- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.
See detailed instructions here:

11) 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.

12) For more information: There is space at the end of each article to list relevant web links for further consultation by our readers. Could you identify some relevant ones and provide such information as well? Some examples are patient associations, relevant databases, OMIM/proteins/genes links, author's websites, etc...

13) Author contributions: CRediT has replaced the traditional author contributions section because it offers a systematic machine readable author contributions format that allows for more effective research assessment. Please remove the Authors Contributions from the manuscript and use the free text boxes beneath each contributing author's name in our system to add specific details on the author's contribution. More information is available in our guide to authors.

14) Disclosure statement and competing interests: 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.

15) 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-sentences bullet points that summarizes the paper. Please write the bullet points to summarize 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.

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.

16) As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://embomolmed.embopress.org/content/2/9/329), EMBO Molecular Medicine will publish online a Review Process File (RPF) to accompany accepted manuscripts.
In the event of acceptance, this file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. Let us know whether you agree with the publication of the RPF and as here, if you want to remove or not any figures from it prior to publication.
Please note that the Authors checklist will be published at the end of the RPF.

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.

I look forward to receiving your revised manuscript.

Yours sincerely,

Lise Roth

Lise Roth, PhD
Senior Editor
EMBO Molecular Medicine

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

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

The VEGFR-PI3K-AKT pathway has been reported to regulate PARPi resistance and CD8+ T cell exhaustion. Moreover, PARPi in combination with VEGFRi (with or without immune checkpoint inhibitors) has been tested in clinical trials. The model systems heavily rely on loss-of-function models.

Referee #1 (Remarks for Author):

In this manuscript, the authors reported that VEGFR1 in breast cancer cells promotes PARPi resistance through AKT pathway activation and CD8+ T cell depletion. I have the following comments:

-The VEGFR-PI3K-AKT pathway has been found to play important roles in PARPi resistance of tumor cells, and inhibitors targeting this pathway combined with PARPi have been evaluated in clinical trials. For example, the combination of olaparib and cediranib, a VEGFR1-3 inhibitor, has been demonstrated to be clinically superior to olaparib monotherapy in recurrent platinum-sensitive ovarian cancer (The Lancet Oncology 2014, PMID: 25218906).

-It has been reported that activation of the VEGFA-VEGFR pathway promotes CD8+ T cell exhaustion in the tumor microenvironment, leading to immunotherapy resistance (e.g., J Exp Med. 2015, PMID: 25601652). The PD-L1 inhibitor, durvalumab, in combination with olaparib and cediranib, has been tested in recurrent women's cancers (J Immunother Cancer 2019, PMID: 31345267). Thus, the novelty of this manuscript seems limited.

-This manuscript mainly relies on loss-of-function experiments. Since the title is "FLT1 activation in tumor cells drives PARP-inhibitor resistance in breast cancer", more data are needed to directly demonstrate this conclusion.

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

While FLT1/VEFG1 has been implicated in driving tumor growth and/or metastasis in other cancers, this appears to be the first time it has been implicated in adaptive resistance to poly (ADP-ribose) polymerase (PARP) inhibitors. Further, in contrast to previous work showing the role of secondary mutations in PARP inhibitor (PARPi) resistance, this work shows a putative role for the PGF-FLT1-AKT pathway in mediating PARPi resistance that is potentially actionable. So along with the models these authors have developed, novelty and medical impact are deemed high and the model systems are adequate.

Referee #2 (Remarks for Author):

This manuscript by Tai et al. (EMM-2023-18653) addresses the issue of mechanisms, aside of secondary mutations in

homology-dependent repair (HDR) genes, in the development of resistance of breast cancers to poly (ADP-ribose) polymerase (PARP) inhibitors despite an initial response. For this purpose, the authors utilize tumor cells from mice with conditional deletion of Brca1 or Bard1, then do orthotopic transplants into mammary glands and develop PARP inhibitor (PARPi) resistance in vivo via exposure to the PARPi talazoparib. PARPi sensitive controls were not exposed to talazoparib. On this basis, the authors find that PARPi resistance in mice is associated with increased expression of placental growth factor (PGF), its ligand FLT1/VEGF, and activation of a downstream target, AKT. There is some corroboration of these effects in human PARPi resistant breast cancers that have mutations in the HDR genes BRCA1, BRCA2 or PALB2. Additionally, the authors show that genetic or pharmacological inhibition of FLT1 in PARPi resistant tumors restores sensitivity and leads to T-cell dependent regression of tumors in immunocompetent mice in the presence of PARPi. Further, the authors demonstrate that in vitro treatment of PARPi resistant tumor cells from mice increases levels of phospho-FLT1 (pFLT1). While the innovative models are appreciated and elucidation of a PGF-FLT1-AKT signaling axis in PARPi resistant breast cancer appears to be novel, and is medically relevant and has translational implications, there are concerns, detailed below, with certain aspects of this study that should be addressed.

Major issues:
1. To support a role for FLT1 in mediating PARPi resistance, in Fig. 3, Flt1 is suppressed using a single gRNA carried by a lentivirus. There is no consideration of possible off-target effects, much less rescue by re-expressing Flt1 or increasing confidence with use of a 2nd, independent, gRNA.

2. While Figs. 2E-F and 5E-F show that both Brca1-deficient and Bard1-deficient tumors that are resistant to PARPi have increased PGF and pAKT, respectively, as compared to PARPi sensitive tumors, further support to corroborate the existence of a PGF-FLT1-AKT1 pathway in PARPi resistance is lacking. For example, under conditions of suppression of Flt1 in PARPi resistant tumors from mice, is pAKT suppressed? Or, if one compares human breast cancers (such as in Figs. 3G-H and 7B that have high pFLT1), do pAKT levels correlate with levels of pFLT1?

3. Although the extension to human breast cancers in Figs. 3G-H and Fig. 7 is important, there are concerns. For example, Fig. 3G indicates that "tissue sections from PARPi-resistant tumors" were utilized in Fig. 3H. However, Fig. 3H stratifies progression-free survival in these PARPi-resistant tumors by high or low levels of pFLT1. If all of the tumors are PARPi-resistant, maybe differences in pFLT1 (FLT1 activation) are not driving resistance but merely controlling the growth rate of the tumors. Can clarification be added to address this point? Also, while Suppl. Tables 1 and 2 indicate that human breast cancer samples utilized in Figures 3G-H and 7, respectively, had mutations in either BRCA1, BRCA2 or PALB2, the Results section and the figure legends do not appear to indicate which of the high vs low pFLT1 tumors had mutations in each gene. This is particularly important since the only extension of the results in mice for Brca1- and Bard1-deficiency to these other key HDR genes (BRCA1 and PALB2) is in these experiments utilizing human tumors. Additionally, the presentation for these human tumors would be strengthened by showing examples (images) of tumors with high vs low pFLT1.

Minor issues:
1. Why, as shown in Figs. 1B&E do Bard1-deficient tumors grow more rapidly than Brca1-deficient tumors in the absence of PARPi?

2. In many figures, such as in Figs. 2B, 2D, 2F and 3B the scales are different, sometimes dramatically so, when comparing Brca1-deficient vs Bard1-deficient cells/tumors in the same figure part. At a minimum, the authors should acknowledge this and give some justification for presenting the data in this way.

3. While both Brca1-deficient and Bard1-deficient tumors show clear differences between the growth/survival of resistant vs sensitive cells in vivo, they argue the differences are less pronounced in vitro (Suppl. Fig. 1). In actuality, in vitro, it looks as though there are clear and significant differences for Brca1-deficient cells but not Bard1-deficient tumor cells. Rather than indicating (as in the title to Suppl. Fig. 1) that both Brca1- and Bard1-deficient cells show "modest differences in talazoparib sensitivity", it might be clearer to acknowledge that these two genotypes do not appear to behave in the same way in vitro.

4. Either the text of the Results section or the legend to figure 2 should clarify how endothelial cells are being identified for this figure, on the basis of being CD31+, by morphology, or by both.

5. Suppl. Figure 2 compares Brca1- and Bard1-deficient tumors that are sensitive or resistant to PARPi for immune cell composition. The authors should acknowledge that for certain types of immune cells, such as B cells and macrophages, that there are not consistent differences between sensitive and resistant tumors in the two distinct genotypes.

6. While Suppl. Fig. 3B quantifies the number of FLT1+ cells in a specific area, based on the examples shown in Supp. Fig. 3A, it would appear that the mean intensity of FLT1 is much higher in PARPi resistant tumors from a Brca1-deficient genetic background than for Bard1-deficient tumors. Either more representative examples are needed or there some clarification should be added.

7. Neither the text of the Results section nor the legend to Fig. 6 appears to indicate what model of T-cell deficient mice is

utilized.

Referee #3 (Comments on Novelty/Model System for Author):

The experiments reported here are mainly performed using in vivo models. As also suggested in my report, the use of organoides derived from BC patients may be an alternative for some experiments. However, I believe that the authors have done a great job and the results are very interesting and with a great potential.

Referee #3 (Remarks for Author):

The authors identify a novel mechanism underlying the resistance to PARPi in breast cancers, that through the activation of the FLT1 and AKT leads to cell survival of BRCA1/2-mutant breast cancer. The experiments are performed mostly in in vivo models, that is remarkable, and strongly support the conclusions drawn in the paper. The text is well-written and straightforward. The topic they face is of great importance. Therefore, overall, I believe that the work presented is suitable for publication in EMM.

However, I have some comments/suggestions and requests, more specifically from a mechanistic point of view.

The data clearly demonstrate that AKT is activated, conferring pro-survival signals. I suppose that the authors checked the activation of the S473 residue (since this ab has been listed in the "Material" section).
1. Can the authors indicate the specific residue?
2. What about the activation of the T308?
3. Does FLT1 directly activate AKT?

On the same line, and considering that the PI3K pathway is a well-recognized mechanism of BC resistance, can the authors clarify is there is any link between these two pathways?

Alpelisib is a well-known drug used in combination with PARPi.
Did the authors checked if alpelisib+PARPi phenocopy the effects given by axitinib+PARPi? And, more importantly, do AKT inhibitors (e.g. capivasertib) phenocopy the effects given by axitinib+PARPi? This would strongly demonstrate that the effects here described are through the FLT1-AKT pathway.
Moreover, are there additional targets of the FLT1 activation that contribute to the effects you describe? Maybe a phospho-proteomic analysis may be helpful.

An additional point I would like to highlight is on the use of the PARP inhibitor. The PARP inhibitor used by the authors is talazoparib, that can block the activity of both PARP1 and PARP2. Recent literature shows that inhibition of PARP2 caused side effects at hematopoietic level; a novel and highly selective PARP1 inhibitor is now available (AZD530). I would suggest to add the use this inhibitor in key experiments.

To further strength their findings, could the author use the proposed therapy in organoids derived from BC patient resistant to PARPi?

Point-by-point response to reviewers (in blue).

Reviewer#1:

The VEGFR-PI3K-AKT pathway has been reported to regulate PARPi resistance and CD8+ T cell exhaustion. Moreover, PARPi in combination with VEGFRi (with or without immune checkpoint inhibitors) has been tested in clinical trials. The model systems heavily rely on loss-of-function models.

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-This manuscript mainly relies on loss-of-function experiments. Since the title is "FLT1 activation in tumor cells drives PARP-inhibitor resistance in breast cancer", more data are needed to directly demonstrate this conclusion.

Authors: We thank the reviewer for the comments. We have now performed additional experiments to address the concerns, and the data has been incorporated into the revised manuscript. The points of similarity and differences with VEGFR-driven ovarian cancer and the novelty of our studies have been clarified in the Discussion. These points are summarized below.

(i) Reliance on loss-of-function experiments and more data needed to support the conclusions: In our revised manuscript, we have incorporated two independent guide RNAs against FLT1 in both mouse models (Brca1- and Bard1-deficient) to further strengthen our conclusions. Our new data (EV3E-F, EV3I and Fig. 3D-E) shows that Flt1 suppression by both gRNAs re-sensitizes PARP inhibitor-resistant tumors to talazoparib treatment in 2 independent models. We further generated FLT1-overexpression constructs to the FLT1-knockdown background for gain-of-function experiments. These experiments showed that FLT1-re-expression in FLT1-suppressed tumors reverses FLT1-suppression-induced tumor regression in the presence of talazoparib in both models (EV3G and 3J-L). Our data support the conclusion that FLT1 is a mediator of PARP-inhibitor resistance in these breast cancer models.

(ii) The known contribution of VEGFR signaling in ovarian cancer, the existing clinical trial data on VEGFR inhibition in ovarian cancer patients and its link to immunotherapy response, which are limiting the novelty of the current study on breast cancer:

We acknowledge this important point raised by the reviewer. The role of VEGFR signaling in tumor growth and during PARP inhibition is indeed well-established in ovarian cancer models and patients, primarily in the context of VEGFR2-driven angiogenesis. In contrast, from our genetic loss- and gain-of-function and VEGFR2-depletion studies, we find that VEGFR1 signaling in cancer cells (rather than VEGFR2-driven angiogenesis) promotes PARP inhibitor resistance in breast cancer. We think this is an important distinction to highlight in our studies, which was written in the Discussion section of our original manuscript and has been further clarified in our revised version. Since pan-VEGFR inhibitors such as cediranib or axitinib do not allow us to differentiate between the contribution of VEGFR1 or VEGFR2, we performed genetic suppression of FLT1 in cancer cells, which allowed

us to identify its novel function in PARP inhibitor resistance in breast cancer for the first time. We compared these results with antibody-mediated depletion of VEGFR2 (abundantly expressed in endothelial cells), which reduced angiogenesis but did not significantly reduce PARP inhibitor resistance in our models. We think this is especially important since FLT1 or VEGFR1 activation in cancer cells is often overlooked in cancer studies. However, this might be clinically relevant since FLT1 activation in cancer cells can be a useful biomarker for screening tumors that might develop PARPi resistance. Our clinical data (Fig. 7) suggests that FLT1 activation in cancer cells could be used to stratify patients who might benefit from VEGFR inhibition. Therefore, the FLT1 pathway could serve both as a biomarker and therapeutic target for overcoming PARPi resistance in breast cancer.

Regarding the existing literature on T-cell exhaustion and immunotherapy resistance with VEGFR, these studies were performed primarily in the context of VEGF-A signaling and do not shed light on the contribution of PGF-FLT1 signaling in cancer cells. In our studies, the genetic blockade of FLT1 in cancer cells allowed us to identify a novel impact on CD8+ T-cell number in breast cancer models. Unlike VEGF-A signaling effects on T-cell exhaustion, we find that blocking PGF-FLT1 signaling in cancer cells increases the CD8+ T-cell numbers in these tumors and promotes tumor regression (Fig. 6A-B and Fig. 6F-G), an effect that is reversed in T-cell-deficient mice (Fig. 6C-E). Our unpublished preliminary data suggest that FLT1 blockade in cancer cells alters the chemokine landscape of tumors, which allows greater recruitment of CD8+ T-cells and induces cytotoxicity in tumors. Thus, depending on the type of VEGF ligands (VEGF-A vs. PGF) and engagement of their respective receptors (VEGFR2 vs. FLT1, respectively), the impact on the immune system can be very different. We have updated the Discussion to clarify these points.

Referee #2 (Comments on Novelty/Model System for Author):
Responses to Reviewer 2 (in blue).

While FLT1/VEFG1 has been implicated in driving tumor growth and/or metastasis in other cancers, this appears to be the first time it has been implicated in adaptive resistance to poly (ADP-ribose) polymerase (PARP) inhibitors. Further, in contrast to previous work showing the role of secondary mutations in PARP inhibitor (PARPi) resistance, this work shows a putative role for the PGF-FLT1-AKT pathway in mediating PARPi resistance that is potentially actionable. So along with the models these authors have developed, novelty and medical impact are deemed high and the model systems are adequate.
Authors: We appreciate the encouraging comments about the novelty of these studies.

Referee #2 (Remarks for Author):

This manuscript by Tai et al. (EMM-2023-18653) addresses the issue of mechanisms, aside of secondary mutations in homology-dependent repair (HDR) genes, in the development of resistance of breast cancers to poly (ADP-ribose) polymerase (PARP) inhibitors despite an initial response. For this purpose, the authors utilize tumor cells from mice with conditional deletion of Brca1 or Bard1, then do orthotopic transplants into mammary glands and develop PARP inhibitor (PARPi) resistance in vivo via exposure to the PARPi talazoparib. PARPi sensitive controls were not exposed to talazoparib. On this basis, the authors find that PARPi resistance in mice is associated with increased expression of placental growth factor (PGF), its ligand FLT1/VEGF, and activation of a downstream target, AKT. There is some corroboration of these effects in human PARPi resistant breast cancers that have mutations in the HDR genes BRCA1, BRCA2 or PALB2. Additionally, the authors show that genetic or pharmacological inhibition of FLT1 in PARPi resistant tumors restores sensitivity and leads to T-cell dependent regression of tumors in immunocompetent mice in the presence of PARPi. Further, the authors demonstrate that in vitro treatment of PARPi resistant tumor cells from mice increases levels of phospho-FLT1 (pFLT1). While the innovative models are appreciated and elucidation of a PGF-FLT1-AKT signaling axis in PARPi resistant breast cancer appears to be novel, and is medically relevant and has translational implications, there are concerns, detailed below, with certain aspects of this study that should be addressed.

Major issues:
1. To support a role for FLT1 in mediating PARPi resistance, in Fig. 3, Flt1 is suppressed using a single gRNA carried by a lentivirus. There is no consideration of possible off-target effects, much less rescue by re-expressing Flt1 or increasing confidence with use of a 2nd, independent, gRNA.

Authors: We appreciate this suggestion by the reviewer and have performed additional experiments to address this concern. Our new data (EV3 E-F, EV3I and Fig. 3D-E) shows that Flt1 suppression by both gRNAs in 2 independent models re-sensitizes PARP inhibitor-resistant tumors to talazoparib treatment. We further generated FLT1-overexpression constructs to the FLT1-knockdown background for gain-of-function experiments in both models. These experiments showed that FLT1-re-expression in FLT1-suppressed tumors reverses FLT1-suppression-induced tumor regression in the presence of talazoparib (EV3G and EV3J-L). Our data support the conclusion that FLT1 is a mediator of PARP-inhibitor resistance in these breast cancer models.

2. While Figs. 2E-F and 5E-F show that both Brca1-deficient and Bard1-deficient tumors that are resistant to PARPi have increased PGF and pAKT, respectively, as compared to PARPi sensitive tumors, further support to corroborate the existence of a PGF-FLT1-AKT1 pathway in PARPi resistance is lacking. For example, under conditions of suppression of Flt1 in PARPi resistant tumors from mice, is pAKT suppressed? Or, if one compares human breast cancers (such as in Figs. 3G-H and 7B that have high pFLT1), do pAKT levels correlate with levels of pFLT1?

Authors: We agree this experiment would be informative. Figs. 2E- F and Figs. 5C-D show increased PGF and pAKT in the PARPi-resistant tumors for both models. We have corroborated these findings in the tumors lacking FLT1 through either genetic (Fig. 5E-F) or pharmacologic (Fig. 5G-H) inhibition of FLT1.

Regarding patient samples from BRCA-deficient patients, these are a rare subset. When pre-treatment biopsies are obtained from these patients, they are prioritized for pathological evaluation, and any residual slides are kept for research. To overcome these limitations, we collaborated with two centers (MSKCC and Emory) to get samples for clinical validation of our findings. We had only two blank sections per patient left over after clinical evaluation, on which we performed phospho- and total-FLT1 immunohistochemistry (Fig. 7 and Appendix Fig. S2). Therefore, we were unable to test pAKT on these samples. We will expand to additional cohorts and institutions in future studies to address these questions.

3. Although the extension to human breast cancers in Figs. 3G-H and Fig. 7 is important, there are concerns. For example, Fig. 3G indicates that "tissue sections from PARPi-resistant tumors" were utilized in Fig. 3H. However, Fig. 3H stratifies progression-free survival in these PARPi-resistant tumors by high or low levels of pFLT1. If all of the tumors are PARPi-resistant, maybe differences in pFLT1 (FLT1 activation) are not driving resistance but merely controlling the growth rate of the tumors. Can clarification be added to address this point?
Authors: We agree this could be confounding unless we have matched pre- and post-treatment tissues. Therefore, we have removed these data from the manuscript.

Also, while Suppl. Tables 1 and 2 indicate that human breast cancer samples utilized in Figures 3G-H and 7, respectively, had mutations in either BRCA1, BRCA2 or PALB2, the Results section and the figure legends do not appear to indicate which of the high vs low pFLT1 tumors had mutations in each gene. This is particularly important since the only extension of the results in mice for Brca1- and Bard1-deficiency to these other key HDR genes (BRCA1 and PALB2) is in these experiments utilizing human tumors.
Authors: We have now provided this information in Table EV1.

Additionally, the presentation for these human tumors would be strengthened by showing examples (images) of tumors with high vs low pFLT1.
Authors: We have now provided this information in Fig. 7B.

Minor issues:
1. Why, as shown in Figs. 1B&E do Bard1-deficient tumors grow more rapidly than Brca1-deficient tumors in the absence of PARPi?
Authors: We agree with the reviewer that there are possibly inherent differences between the two tumor cell lines in growth patterns, which we have not explored. For instance, *Bard1*-def tumors induce cachexia (our published studies, PMID 32730698) whereas *Brca1*-def tumors do not (mentioned in the Results section). At present, we do not know the underlying mechanisms behind these differences.

2. In many figures, such as in Figs. 2B, 2D, 2F and 3B the scales are different, sometimes dramatically so, when comparing Brca1-deficient vs Bard1-deficient cells/tumors in the same figure part. At a minimum, the authors should acknowledge this and give some justification for presenting the data in this way.

Authors: The scales change for each figure to make it easier for the reader to visualize the fold change between experimental and control groups. The data is normalized to the control group, thus setting the control to 1 in each case. The values for each of the experimental groups are compared relative to their respective control groups. The magnitude of the scale is reflective of the relative expression of the experimental group over control expression. We have clarified this now in the Methods section.

3. While both Brca1-deficient and Bard1-deficient tumors show clear differences between the growth/survival of resistant vs sensitive cells in vivo, they argue the differences are less pronounced in vitro (Suppl. Fig. 1). In actuality, in vitro, it looks as though there are clear and significant differences for Brca1-deficient cells but not Bard1-deficient tumor cells. Rather than indicating (as in the title to Suppl. Fig. 1) that both Brca1- and Bard1-deficient cells show "modest differences in talazoparib sensitivity", it might be clearer to acknowledge that these two genotypes do not appear to behave in the same way in vitro.
Authors: We have updated the title and results section to reflect this point.

4. Either the text of the Results section or the legend to figure 2 should clarify how endothelial cells are being identified for this figure, on the basis of being CD31+, by morphology, or by both.
Authors: We have updated the figure legend to indicate that the endothelial cells are being identified by both CD31 immunostaining and morphology.

5. Suppl. Figure 2 compares Brca1- and Bard1-deficient tumors that are sensitive or resistant to PARPi for immune cell composition. The authors should acknowledge that for certain types of immune cells, such as B cells and macrophages, that there are not consistent differences between sensitive and resistant tumors in the two distinct genotypes.
Authors: We have updated the Results section to address this point.

6. While Suppl. Fig. 3B quantifies the number of FLT1+ cells in a specific area, based on the examples shown in Supp. Fig. 3A, it would appear that the mean intensity of FLT1 is much higher in PARPi resistant tumors from a Brca1-deficient genetic background than for Bard1-deficient tumors. Either more representative examples are needed or there some clarification should be added.
Authors: Since our text appears confusing, we would like to clarify a few points. We have updated this in our Methods section as well.
(a) In Fig 3A-B, EV3A-B and all figures with image analysis, we have quantified the entire tissue sections (and not specific fields) from all mice shown using the Qu Path software.
(b) The Qu Path software used in this study enables the quantification of the signal intensity of a particular immunostaining across the entire section. Using this software, the positive cells of the selected signal intensity are quantified. The final data were generated by dividing the number of positive cells (of the selected staining intensity) by the total area of the tumor section, and the results are normalized to the comparator to present the data as a fold change relative to their respective control (which is set to 1). These images and staining intensities are manually examined by a trained pathologist.
Therefore, the intensity differences between *Brca1*- or *Bard1*- deficient tumors do not impact the quantification, since the intensities of the experimental groups are compared to their own respective controls within each model. This is also described in detail in the Methods section.

7. Neither the text of the Results section nor the legend to Fig. 6 appears to indicate what model of T-cell deficient mice is utilized.
Authors: We have used the athymic nu/nu (nude-*Foxn1*^{nu}) strain, which is now indicated in the Results and Methods section.

Referee #3 (Comments on Novelty/Model System for Author):
Responses to Reviewer 3 (in blue).

The experiments reported here are mainly performed using in vivo models. As also suggested in my report, the

use of organoides derived from BC patients may be an alternative for some experiments. However, I believe that the authors have done a great job and the results are very interesting and with a great potential.

Referee #3 (Remarks for Author):

The authors identify a novel mechanism underlying the resistance to PARPi in breast cancers, that through the activation of the FLT1 and AKT leads to cell survival of BRCA1/2-mutant breast cancer. The experiments are performed mostly in in vivo models, that is remarkable, and strongly support the conclusions drawn in the paper. The text is well-written and straightforward. The topic they face is of great importance. Therefore, overall, I believe that the work presented is suitable for publication in EMM.
Authors: We appreciate these encouraging comments on the novelty and importance of these *in vivo* drug resistance modeling studies.

However, I have some comments/suggestions and requests, more specifically from a mechanistic point of view.

The data clearly demonstrate that AKT is activated, conferring pro-survival signals. I suppose that the authors checked the activation of the S473 residue (since this ab has been listed in the "Material" section).
1. Can the authors indicate the specific residue?
Authors: We thank the reviewer for pointing out this missing information. The pAKT residue is indeed Ser 473, and we have now updated the figures to make this obvious to the reader.

2. What about the activation of the T308?
Authors: We saw a similar activation of Thr308 in resistant tumors by immunostaining analysis (shown in *Reviewer's only Fig. 1* at the end of this document).

3. Does FLT1 directly activate AKT? On the same line, and considering that the PI3K pathway is a well-recognized mechanism of BC resistance, can the authors clarify is there is any link between these two pathways?
Authors: There is prior literature showing FLT1 binding to the p85 subunit of PI3K *in vitro* (PMID: 7657594). Therefore, it is possible that FLT1 activation of AKT is not direct and could act through PI3K. In future studies, we will determine the binding partners of FLT1, explore whether FLT1 directly binds to PI3K or AKT and identify the upstream and downstream signaling mechanisms to AKT. Our present study is focused on demonstrating that PGF-induced FLT1 activation in cancer cells promotes PARP inhibitor resistance in breast cancer models. We will mechanistically address how FLT is activated in future follow-up studies.

Alpelisib is a well-known drug used in combination with PARPi.
Did the authors checked if alpelisib+PARPi phenocopy the effects given by axitinib+PARPi? And, more importantly, do AKT inhibitors (e.g. capivasertib) phenocopy the effects given by axitinib+PARPi? This would strongly demonstrate that the effects here described are through the FLT1-AKT pathway.
Authors: We previously informed the editor that the syngeneic mouse strain used for implanting the *Brca1*- and *Bard1*-deficient cells had been unavailable for several months, which caused the delay in our revision experiments. In the end, we were only able to obtain age- and sex-matched mice in limited quantities. Therefore, we prioritized the most essential experiments for this revision. Following the reviewer's recommendations, we performed in-vivo tumor assays with the AKT inhibitor (capivasertib) shown below in *Reviewer's only Figure. 2* at the end of this document.

Consistent with the FLT1 inhibition phenotypes, blockade of AKT in combination with PARP inhibitor results in tumor regression of PARP-inhibitor-resistant tumors in the *Brca1*-deficient model. However, we did not have sufficient mice to repeat these experiments in the *Bard1*-deficient model.

Moreover, are there additional targets of the FLT1 activation that contribute to the effects you describe? Maybe a phospho-proteomic analysis may be helpful.
Authors: We appreciate the suggestion and plan to conduct phospho-proteomic analysis in future studies to identify other targets of FLT1 activation beyond AKT.

An additional point I would like to highlight is on the use of the PARP inhibitor. The PARP inhibitor used by the authors is talazoparib, that can block the activity of both PARP1 and PARP2. Recent literature shows that inhibition of PARP2 caused side effects at hematopoietic level; a novel and highly selective PARP1 inhibitor is now available (AZD530). I would suggest to add the use this inhibitor in key experiments.
Authors: We appreciate this insightful comment. In our studies, since resistance to PARP inhibitors is generated *in vivo* by long term daily treatment of mice, we would need to start from the beginning with the new drug AZD530 by first generating PARP inhibitor in-vivo resistance models. This process along with the subsequent validation of the targets could take anywhere from 1-2 years for completion. Therefore, we feel this particular experiment is out of scope for the present study but will be important to perform in future follow-up translational studies.

To further strength their findings, could the author use the proposed therapy in organoids derived from BC patient resistant to PARPi?
Authors: Since the patient samples from BRCA1/2-mutant breast cancer patients are relatively rare, organoid models are not available. Fresh tumor samples are difficult to obtain, which further limits the possibilities of readily generating organoid models, which we agree would be very useful resources for validation.

Additional points:

Please see Figs 1 and 2 (reviewers only) in the next pages.
Title of the manuscript was updated.
A new co-author was added (Yifan Gu, technician in the Acharyya laboratory) to assist with the revision experiments.

REVIEWERS: Figure 1

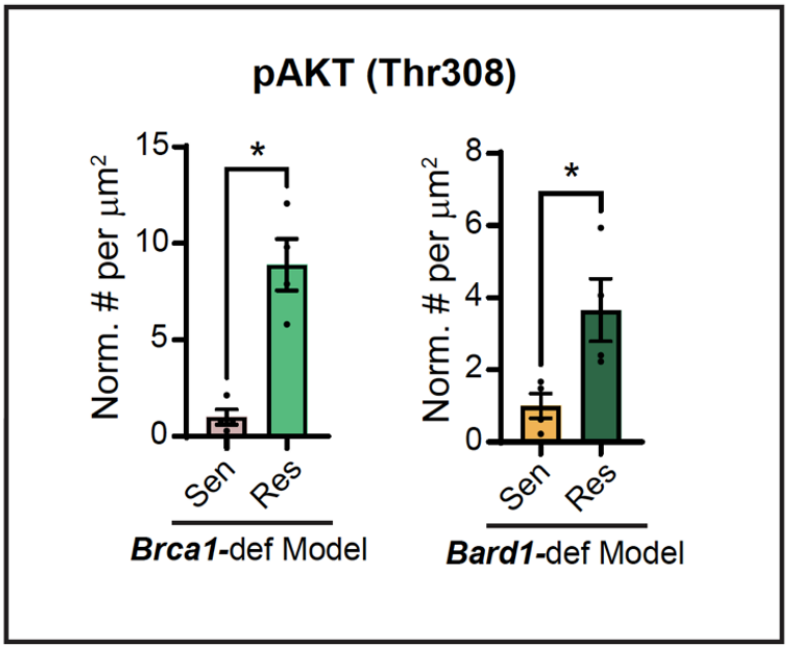

Figure 1. Activation of AKT (Thr 308) in PARPi-resistant *Brca1*- and *Bard1*-def breast tumors. pAKT (Thr308) staining was quantified using automated QuPath software to identify positively stained cells in the indicated tumor sections. *n* = 4 for Sen and Res tumors for both *Brca1*-def and *Bard1*-def models. Data are presented as mean values ± SEM. P values were determined by a two-tailed, unpaired, Mann–Whitney test: * indicates *P* = 0.0286 for both models.

REVIEWERS: Figure 2

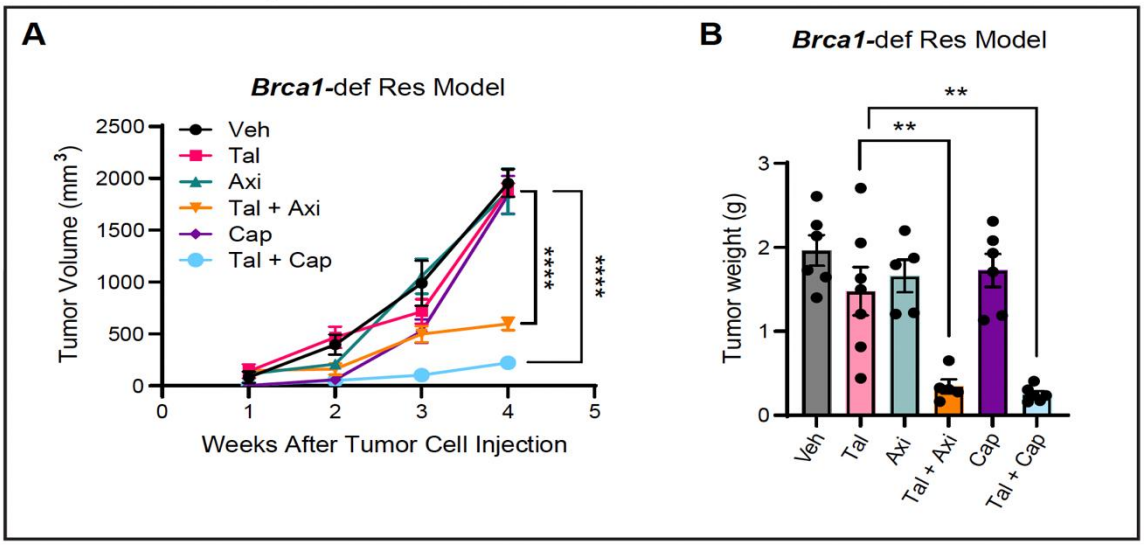

Figure 2. Pharmacological inhibition of AKT with Capivasertib with Talazoparib phenocopies the tumor-inhibitory effect of axitinib+talazoparib treatment. A. Mice were injected with the Tal-resistant ("Res") *Brca1*-def breast tumor cells described in Fig. 1 and then randomized into the following six treatment groups at 2 weeks post tumor-cell injection: 1) vehicle ("Veh"),2) talazoparib ("Tal"), 3) axitinib ("Axi"), 4) Tal + Axi, 5) Capivasertib ("Cap"), and 6) Tal + Cap. Tumor size was measured weekly to monitor tumor growth, and mice were euthanized for tumor collection at 4 weeks post tumor-cell injection. *n* = 6 Veh-treated tumors, *n* = 7 Tal-treated tumors, *n* = 5 Axi treated tumors, *n* = 5 tumors treated with Tal + Axi, *n* = 6 Cap-treated tumors, and *n* = 6 tumors treated with Tal + Cap. Veh, Tal, Axi and Tal+ Axi data shown from Fig. 4 and EV4. Data are presented as mean values ± SEM. P values were determined with a one-way ANOVA test. The endpoint tumor volume between the Tal and Tal + Axi groups is **** at 4 weeks, indicating $P < 0.0001$. The endpoint tumor volume between the Tal and Tal + Cap groups is **** at 4 weeks, indicating $P < 0.0001$. **B**, Tumor weights from A were plotted at endpoint. *n* = 6 Veh-treated tumors, *n* = 7 Tal-treated tumors, *n* = 5 Axi-treated tumors, *n* = 5 tumors treated with Tal + Axi, *n* = 6 Cap-treated tumors, and *n* = 6 tumors treated with Tal + Cap. Data are presented as mean values ± SEM. P values were determined by a two tailed, unpaired, Mann-Whitney test. ** indicated $P = 0.0051$ between the Tal and Tal + Axi groups and **indicates $P = 0.0012$ between the Tal and Tal + Cap groups.

29th May 2024

Dear Dr. Acharyya,

Thank you for submitting your revised study. Your manuscript was sent back to the initial referees. Referee #2 was unfortunately not available, but referee #3 also evaluated your answers to this referee's concerns. As you will see below, they are satisfied with the revisions, and I will therefore be able to accept your manuscript once the following editorial points will be addressed:

1/ Manuscript text:
- Please remove the coloured font, and only keep in track changes mode any new modification.
- We can accommodate a maximum of 5 keywords, please adjust accordingly.
- Please remove "data not shown" (p.6): as per our guidelines on "Unpublished Data" the journal does not permit citation of "Data not shown". All data referred to in the paper should be displayed in the main or Expanded View figures.
- Methods:
o Patient samples: please include a statement that informed consent was obtained from all subjects.
o Please add a Graphics section, mentioning the use of Biorender: "(some of the... OR Figure #... OR synopsis) Graphics were created with BioRender.com".
- Data availability: please remove the current text and indicate "This study includes no data deposited in external repositories." (see also: https://www.embopress.org/page/journal/17574684/authorguide#availabilityofpublishedmaterial)
- The acknowledgement section should be placed after the Methods section, and match the information provided in the submission system (currently HICCC Pilot Awards, Columbia University Irving Scholars Program (and P30CA013696?) are missing in the submission system).
- A "Disclosure statement and competing interests" section should be included after the acknowledgements (https://www.embopress.org/competing-interests).
- The References should be listed in alphabetical order, with 10 author names before et al.

2/ Figures and Appendix:
- Please address the queries from our data editors in the figure legends:
1. Although 'n' is provided, please describe the nature of entity for 'n' in the legends of figures 2b, d, f, h, j; 3b; 5c, f; 6b-c, e, g, j; EV 2a-d; EV 3b, d-i, k-l; EV 5d.
2. Please note that the error bars are not defined in the legends of figures 1c, f; EV 2c.
3. Please note that the scale bar needs to be defined for figures 2a, c, e, g; 3a; 5c, e, g; 6a, f; 7b; EV 3a, c.
- Appendix: please add page numbers (including in the table of content). Ideally the legends should be placed underneath the corresponding figure and table. Table should be renamed "Appendix Table S1"
- Thank you for providing Source Data. Please check the provided SD values for Figure 3D, Flt1i (gRNA1) + Veh (week 1 and 2 identical).

4/ I introduced minor modifications to your synopsis, let me know if you agree with the following or amend as you see fit:

PARP inhibitor (PARPi) resistance is a major treatment challenge that dramatically shortens patient survival. Using new mouse models of PARPi response and recurrence, we identified FLT1 as a potential biomarker and therapeutic target for reversing PARPi resistance in BRCA deficient-breast cancer.
• New mouse models were developed that recapitulate the PARPi response and recurrence observed in patients.
• A novel PARPi-adaptive resistance mechanism driven by the PGF-FLT1-AKT pathway was identified.
• FLT1 signaling protected the cells from PARPi-induced death by activating AKT pro-survival pathways and by dampening the cytotoxic immune response.
• Blocking FLT1 signaling, either genetically or pharmacologically using axitinib, re-sensitized PARPi-resistant tumors to PARPi treatment in mice.
• High FLT1 activation in tumor cells at pre-treatment significantly correlated with shorter progression-free survival on PARPi in patients with breast cancer.

Thank you for providing a nice synopsis image. Please resize it to 550 px wide x 300-600 px high and make sure that the text remains legible.

5/ As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://embomolmed.embopress.org/content/2/9/329), EMBO Molecular Medicine will publish online a Review Process File (RPF) to accompany accepted manuscripts.
This file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. Let us know whether you agree with the publication of the RPF and as here, if you want to remove or not any figures from it prior to publication.
Please note that the Authors checklist will be published at the end of the RPF.

I look forward to receiving your revised manuscript.

With kind regards,

Lise Roth

Lise Roth, PhD
Senior Editor
EMBO Molecular Medicine

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

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

The revised manuscript addressed the critiques. The models used are adequate.

Referee #1 (Remarks for Author):

The authors have addressed my previous comments. I have no additional concerns.

Referee #3 (Remarks for Author):

The authors addressed most of the points raised by reviewers. They have perform a meticulous revision of the manuscript, providing strong evidence of the importance of FLT1 activation in promoting resistance to PARPi in BC. I believe the manuscript is suitable for publication in EMM Journal.

June 4, 2024

Dear Dr. Roth,

Thank you for giving us the opportunity to resubmit our manuscript. We have addressed all the points that you mentioned below, and the point-by-point responses are included.

Thank you,
Swarnali

1/ Manuscript text:
- Please remove the coloured font, and only keep in track changes mode any new modification.
Authors: We have made these changes.

- We can accommodate a maximum of 5 keywords, please adjust accordingly.
Authors: We have made these changes and updated the manuscript.

- Please remove "data not shown" (p.6): as per our guidelines on "Unpublished Data" the journal does not permit citation of "Data not shown". All data referred to in the paper should be displayed in the main or Expanded View figures.
Authors: we have removed them.

- Methods:
o Patient samples: please include a statement that informed consent was obtained from all subjects.
Authors: Completed.

o Please add a Graphics section, mentioning the use of Biorender: "(some of the... OR Figure #... OR synopsis) Graphics were created with BioRender.com".
Authors: Completed.

- Data availability: please remove the current text and indicate "This study includes no data deposited in external repositories." (see also: https://www.embopress.org/page/journal/17574684/authorguide#availabilityofpublishedmaterial)
Authors: Completed.

- The acknowledgement section should be placed after the Methods section, and match the information provided in the submission system (currently HICCC Pilot Awards, Columbia University Irving Scholars Program (and P30CA013696?) are missing in the submission system).
Authors: The relocation of the acknowledgement section has been done. All grants supporting the study and additional ones (supporting my salary during this study) have been added.

- A "Disclosure statement and competing interests" section should be included after the acknowledgements (https://www.embopress.org/competing-interests).
Authors: Completed.

- The References should be listed in alphabetical order, with 10 author names before et al.
Authors: They have been updated using Endnote (EMBO Mol Med Style).

2/ Figures and Appendix:
- Please address the queries from our data editors in the figure legends:
1. Although 'n' is provided, please describe the nature of entity for 'n' in the legends of figures 2b, d, f, h, j; 3b; 5c, f; 6b-c, e, g, j; EV 2a-d; EV 3b, d-i, k-l; EV 5d.
Authors: Updated.

2. Please note that the error bars are not defined in the legends of figures 1c, f; EV 2c.
Authors: Updated.

3. Please note that the scale bar needs to be defined for figures 2a, c, e, g; 3a; 5c, e, g; 6a, f; 7b; EV 3a, c.
Authors: Updated.

- Appendix: please add page numbers (including in the table of content). Ideally the legends should be placed underneath the corresponding figure and table. Table should be renamed "Appendix Table S1"
Authors: Updated.

- Thank you for providing Source Data. Please check the provided SD values for Figure 3D, Flt1i (gRNA1) + Veh (week 1 and 2 identical).
Authors: It is the same for both. We checked the data again.

4/ I introduced minor modifications to your synopsis, let me know if you agree with the following or amend as you see fit:

PARP inhibitor (PARPi) resistance is a major treatment challenge that dramatically shortens patient survival. Using new mouse models of PARPi response and recurrence, we identified FLT1 as a potential biomarker and therapeutic target for reversing PARPi resistance in BRCA deficient-breast cancer.
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• High FLT1 activation in tumor cells at pre-treatment significantly correlated with shorter progression-free survival on PARPi in patients with breast cancer.
Authors: We agree to these changes.

Thank you for providing a nice synopsis image. Please resize it to 550 px wide x 300-600 px high and make sure that the text remains legible.
Authors: This was completed.

5/ As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://embomolmed.embopress.org/content/2/9/329), EMBO Molecular Medicine will publish online a Review Process File (RPF) to accompany accepted manuscripts.
This file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. Let us know whether you agree with the publication of the RPF and as here, if you want to remove or not any figures from it prior to publication.
Please note that the Authors checklist will be published at the end of the RPF.
Authors: We agree to this and there is no restriction on the figures or texts to be published online.

7th Jun 2024

Dear Dr. Acharyya,

Thank you for sending your revised files. I am 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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Should you be planning a Press Release on your article, please get in contact with embo_production@springernature.com as early as possible in order to coordinate publication and release dates.

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

With kind regards,

Lise

Lise Roth, Ph.D
Senior Editor
EMBO Molecular Medicine

>>> Please note that it is EMBO Molecular Medicine 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. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: https://www.embopress.org/transparent-process#Review_Process

EMBO Press Author Checklist

Corresponding Author Name: Swarnali Acharyya	
Journal Submitted to: EMBO Molecular Medicine	
Manuscript Number: EMM-2023-18653-V2	

USEFUL LINKS FOR COMPLETING THIS FORM
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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your

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
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Methods, Data availability

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	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	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.	Yes	Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Methods

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	Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	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.	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.	Yes	Table EV1

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	Methods and Acknowlegdement

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 has extensive information
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	Figure legends has extensive information + Methods
Include a statement about **blinding** even if no blinding was done.	Yes	Figure Legends 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.	Yes	Pre-established criteria set. For instance- since cachexia develops in the Bard1 def-model, we had set the collection timepoint based on our previous cachexia studies and that was followed through out the study. This has been mentioned in the Results Section.
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	Yes. These were performed in consultation with Dr. Manoj Kandpal (author on the manuscript) with biostatistical expertise. Biostatistical section and figure legend includes recommended tests used.

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	Three times. Methods.
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Yes. Figure Legends and raw data has the details.

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.	Yes	Approved IRBs from MSKCC and Emory University. Authors at Columbia only obtained deidentified information.
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.	Yes	Methods
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	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	

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	No such data were generated and this has been stated in the Data Availability Statement after the Methods section in the manuscript.
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	No such data were generated and this has been stated in the Data Availability Statement after the Methods section in the manuscript.
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	No such data were generated and this has been stated in the Data Availability Statement after the Methods section in the manuscript.
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	No such data were generated and this has been stated in the Data Availability Statement after the Methods section in the manuscript.