EMBO *reports*

Differential roles of ERRFI1 in EGFR and AKT pathway regulation affect cancer proliferation

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Transaction Report: This manuscript was transferred to *EMBO reports* following review at *The EMBO Journal*

(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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 6 July 2017

Thank you for the transfer of your research manuscript to EMBO reports. I now have read you're your manuscript and went through the referee reports from The EMBO Journal.

All referees acknowledge the potential interest of the findings. Nevertheless, the three referees have raised a number of concerns and suggestions to further improve the manuscript, or to strengthen the data and the conclusions drawn. As the reports are below, I will not detail them here.

As EMBO reports emphasizes novel functional over detailed mechanistic insight, we will not require to address the points regarding more mechanistic details experimentally, e.g. point 7 of referee #1, or points 1 and 4 of referee #2, and point 5 of referee #3 (of course, in case you already have such date, we ask you to add these to your manuscript). Nevertheless, please address these points in your rebuttal letter, and by adjustments or further discussion in the manuscript text.

Important, though, would be to address the major point by referee #1 by further data, i.e. to prove that the ERRFI1-mediated effect on promoting Akt signaling plays a role in the tumorigenesis process in vivo. Further, please address the remaining concerns regarding the experimental designs, inconsistencies in the results and data presentation raised by the referees.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be fully addressed in the revised manuscript (as detailed above) and in a complete point-by-point response. Acceptance of your manuscript will depend on a positive outcome of the next round of review and will depend on the completeness of

your responses.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Please refer to our guidelines for preparing your revised manuscript:

http://embor.embopress.org/authorguide#manuscriptpreparation

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Important: All materials and methods should be included in the main manuscript file.

Regarding data quantification and statistics, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information must be provided in the figure legends. Please provide statistical testing where applicable.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

This revised manuscript by Cairns et al. described a study on identifying ERRFI1 as a positive regulator of Akt phosphorylation in EGFR low cells. The quality of this version of the manuscript has been largely improved after incorporating quantification of data in multiple figures. The explanation on why ERRFI1 may have seemingly opposite functions depending on EGFR expression is better described as well. However, since a number of in vivo studies using ERRFI1 knockout mice have clearly demonstrated the tumor suppressor role of ERRFI in multiple tumor models, it is difficult to envision if this ERRFI1-mediated effect on promoting Akt signaling plays any role in the tumorigenesis process. Given the results that ERRFI1 only promotes Akt phosphorylation when EGFR is low, but there is no clear classification of what is considered low vs. high, the implication of this study is likely limited.

Specific comments:
1) The relevance of GWAS results generated in LCLs remains questionable given the fact that the rest of the studies used non-LCL cells.

2) Figure 2B, the relative expression of ERRFI1 needs to be included in the analysis.

3) Figure 3C, western blots of input proteins need to be included.

4) The quantification results shown in the bar graphs in Figure 4C and 4D are problematic. Apparently, the western blots shown in Figure 4A were generated from different cell lines and those proteins were not run on the same gel. Thus, the quantified results cannot be all normalized to HCT116. Results from each cell line need to be normalized within itself, or all the proteins need to be run on the same gel and exposed for the same amount of time.

5) The input blots in Figure 5E was supposedly from U251 cells transfected with different ERRFI1 constructs. However, most of the blots (expect p473 blot) were the same as those shown in the original version of the manuscript, in which the data were generated from transfected 293T cells.

6) Figure 6, the quantified results shown in the bar graph should not be all normalized to MDA-MB-468 cells since individual western blots generated from different cell lines were done separately (see comment #4).

7) The mechanism of why ERRFI1 antagonizes Akt-PHLPP interaction is still not clear. If high levels of EGFR sequester ERRFI1 away from Akt, then overexpression of ERRFI1 would overcome this in EGFR high cells as well.

8) There are a number of previously published studies showing appreciable levels of EGFR expression in both U251 and HCT116 cells. This brings back the critical question: what level of EGFR expression is considered low? A clear definition is not provided.

Referee #2:

Cairns et al. provide molecular insight into the dual role of ERRFI1 in regulation of the EGFR and AKT pathway. The study shows that ERRFI1, an interaction partner and negative regulator of EGFR, also forms a complex with PHLPP and AKT to positively regulate AKT signaling by preventing PHLPP access to AKT. ERRFI1 predominantly interacts with EGFR in cells expressing high levels of EGFR and downregulation of ERRFI1 results of increased AKT phosphorylation and signaling. In contrast, AKT phosphorylation was reduced after ERRFI1 knock down in EGFR-low cell lines and as a result, depletion of ERRFI1 suppresses cell growth and sensitizes cells to chemotherapy.
The authors invested a considerable effort to address my comments and criticism and substantially improved the writing/comprehensiveness of the manuscript. Nearly all my suggestions and comments were addressed in a satisfactory manner and only few points remain unclear (see below). The present study is thorough, interesting and well suited for The Embo Journal.

Points, one should still address:
(1) With the new experiments, the question arises how ERRFI1, Akt and PHLPP interact with each other. If the authors failed to obtain recombinant PHLPP protein to determine whether ERRFI1 directly interacts with PHLPP they could immunoprecipitate ERRFI1 in Akt2 and Akt3 knock-down cells. If PHLPP is no longer co-IPed, the findings would be a strong indication that PHLPP is in the protein complex with AKT and not directly interacting with ERRFI1.
(2) Figure 3B: The Coomassie blue panel seems to have been flipped - the proteins to not correspond to the lane description.
(3) Page 11: The authors write: "Indeed, in EGFR-low cell, overexpression of the CRIB domain deletion construct (ΔCRIB), compared with the full length (FL) construct, decreased AKT phosphorylation (Fig 5E, input), and thus abolished the effect caused by overexpression of full length ERRFI1 on TCN and gemcitabine responses (Fig 5F,$p<0.05$)." A decrease in AKT phosphorylation in Figure 5E is not visible, while the same levels are visible as in the empty vector control. Therefore, it would be better write "...overexpression of the CRIB domain deletion construct (ΔCRIB), compared with the full length (FL) construct, fails to increase AKT phosphorylation...".
(4) Since the efficiency of the different anti-Akt antibodies for immunoprecipitating the different Akt isoform differs, it is difficult to draw the conclusion that Akt2 and Akt3 interact with ERRFI1 better than Akt1. The authors need to show that the antibodies against the three Akt family members are precipitating the Akt members equally well (e.g. by analyzing the supernatant of the

precipitation to show that in all cases the majority of the Akt protein can be precipitated). Alternatively, they tone down their claim.

Referee #3:

In the revised manuscript the authors addressed several of my specific comments. However, some of my comments were not addressed:

1. As I have indicated in the previous review, the first part of the manuscript is not connected to the second part. It is not clear why the authors were looking at SNPs, and because TCN is not a specific Akt inhibitor, changes in gene expression in the first part of the manuscript may have nothing to do with Akt activity.
2. The authors cannot use the term "relative survival" in proliferation assays (Figs. 5F, 7, 8, EV3 and EV5C).
3. The authors should show level of proteins after knockdown (not only RNA).
4. It is not clear why EGFR signaling is not elevated in low EGFR expressing cells after ERRFI1 KD.
5. Since Akt phosphorylated only on Thr308 is sufficient to phosphorylate GSK3, the authors should show the effect on other Akt targets such as FOXO and PRAS40.

1st Revision - authors' response 6 October 2017

Reviewer # 1:

1. *The relevance of GWAS results generated in LCLs remains questionable given the fact that the rest of the studies used non-LCL cells.*

We use a genome wide approach using drug as a probe with the assumption that this approach might help us to identify new candidates involved in AKT regulation. LCLs have been used successfully in many pharmacogenomic studies to identify and understand drug mechanisms and variation in response to a drug (Reference 26, 33, and 55). This cell line system is merely an *in vitro* model system for us to identify potential pharmacogenomics candidate genes. We understand that nongenetic factors might confound the results of these association studies, and since gene regulation is tissue specific, therefore, all of our functional studies to characterize those candidate genes were performed in human tumor cell lines. We have added more information on Page 6. A related question was also asked by Reviewer #3 in Q1, please see our answer.

2. *Figure 2B, the relative expression of ERRFI1 needs to be included in the analysis.*

As suggested by the Reviewer, we added the blot for ERRFI1 levels in the 13 human cancer cell lines screened in Figure 2B.

3. *Figure 3C, western blots of input proteins need to be included.*

As suggested by the Reviewer, we have added the input blots in Figure 3C.

4. *The quantification results shown in the bar graphs in Figure 4C and 4D are problematic. Apparently, the western blots shown in Figure 4A were generated from different cell lines and those proteins were not run on the same gel. Thus, the quantified results cannot be all normalized to HCT116. Results from each cell line need to be normalized within itself, or all the proteins need to be run on the same gel and exposed for the same amount of time.*

As suggested by the Reviewer, the quantification results shown in the bar graphs in Figure 4B, 4C and 4D are now normalized within each cell line, and we have modified the figure legends for the normalization on Page 38 in revised manuscript.

5. *The input blots in Figure 5E was supposedly from U251 cells transfected with different ERRFI1 constructs. However, most of the blots (expect p473 blot) were the same as those*

shown in the original version of the manuscript, in which the data were generated from transfected 293T cells.

As suggested by the Reviewer, we have updated the input blots in Figure 5E that reflect the data generated in U251 cells.

6. *Figure 6, the quantified results shown in the bar graph should not be all normalized to MDA-MB-468 cells since individual western blots generated from different cell lines were done separately (see comment #4).*

As suggested by the Reviewer, we have normalized results shown in the bar graphs in Figure 6 within each cell line. We have modified the figure legend to clarify the normalization on Page 39 in our revised manuscript.

8. *There are a number of previously published studies showing appreciable levels of EGFR expression in both U251 and HCT116 cells. This brings back the critical question: what level of EGFR expression is considered low? A clear definition is not provided.*

U251 and HCT116 cells express detectable EGFR. The high and low is relative. Based on publicly available RNAseq data (new Dataset EV2) and the availability of cell lines in our lab, we screened 13 cancer cell lines for EGFR protein levels (Figure 2B in the revised manuscript), and then chose cell lines from the extremes of the distribution of EGFR protein levels. In this study, we chose U251 and HCT116 as EGFR-low cell lines to compare to EGFR-high cell lines. Obviously we do not have precise quantification of EGFR levels to set up cutoff in this study. More studies, either experimentally or computationally or both are required to dynamically assess the relationship between EGFR, ERRFI1 and AKT and EGFR activation. We have added more discussion on Page 18.

Reviewer # 2:

2. *Figure 3B: The Coomassie blue panel seems to have been flipped - the proteins to not correspond to the lane description.*

We have corrected the Coomassie blue panel in Figure 3B.

3. *Page 11: The authors write: "Indeed, in EGFR-low cell, overexpression of the CRIB domain deletion construct (ΔCRIB), compared with the full length (FL) construct, decreased AKT phosphorylation (Fig 5E, input), and thus abolished the effect caused by overexpression of full length ERRFI1 on TCN and gemcitabine responses (Fig 5F,$p<0.05$)." A decrease in AKT phosphorylation in Figure 5E is not visible, while the same levels are visible as in the empty vector control. Therefore, it would be better written "...overexpression of the CRIB domain deletion construct (ΔCRIB), compared with the full length (FL) construct, fails to increase AKT phosphorylation..."*

As suggested by the Reviewer, we have modified the sentence in the revised version on Page 10.

Reviewer # 3:

1. *As I have indicated in the previous review, the first part of the manuscript is not connected to the second part. It is not clear why the authors were looking at SNPs, and because TCN is not a specific Akt inhibitor, changes in gene expression in the first part of the manuscript may have nothing to do with Akt activity.*

As stated in our Introduction, our intent to perform GWAS is to use pharmacogenomics as an approach to probe potential new candidate genes that might play a role in regulation of AKT pathway. The advantage of scanning through the genome is that we might have opportunity to identify new AKT regulators or new mechanisms involved in AKT regulation. We agree with the reviewer that the drug is not a specific AKT inhibitor. Therefore, we have stepwise validation

approaches to start with 18 candidate genes to determine their functional impact on both drug cytotoxicity as well as AKT phosphorylation phenotypes.
Previously, we have used similar approach and the same cell line system to identify biomarkers that likely contribute to the variation in drug response and potential biological or signaling pathways by which these biomarkers might affect (References #54, 32, and 20). Through our stepwise approach to analyze SNPs, gene expression and cytotoxicity association as summarized in Figure 1F, we were able to focus on 4 genes which had significant impact on cytotoxicity phenotype when knocked down in cancer cell lines (Figs EV1 and 2) and (Table 1). Our hypothesis is that one of the mechanism by which those genes might change response to TCN is through the regulation of AKT. Therefore, we performed experiments to determine AKT phosphorylation after manipulating these 4 gene levels in cancer cells to identify ERRFI1. It is logical for us to go through the process in order to identify the potential candidate and as stated earlier, by performing GWAS, we might identify novel regulators or new mechanisms involved in AKT regulation, which turns out to be true in this case.

2. *The authors cannot use the term "relative survival" in proliferation assays (Figs. 5F, 7, 8, EV3 and EV5C).*

Figure 5F, 8, EV1, EV2, EV3 and EV5C were cytotoxicity assays to determine drug sensitivity, and the Y axis represent survival fraction at each dose point post-treatment. We have change the label to "Survival fraction" and clarified this in the figure legend on Page 39, Page 40 and Page 43-44. Figure 7 were cell proliferation assays, and we have also clarified on Page 39-40.

3. *The authors should show level of proteins after knockdown (not only RNA).*

We have added the level of proteins to show knockdown efficiency for VEZT, GOLGA8B, ERRFI1, and SLC7A5 in Figure 2A.

4. *It is not clear why EGFR signaling is not elevated in low EGFR expressing cells after ERRFI1 KD.*

In this study, we showed that ERRFI1 functions as a negative regulator of EGFR signaling in EGFR-high cancer cells, a well-known mechanism (Reference 23), resulting in reduced cell proliferation and sensitivity to gemcitabine (Figs 7A and 8). While in EGFR-low cancer cells, we found that ERRFI1 mainly interacts with AKT as a positive regulator of AKT Ser473 by regulating the interaction between AKT and PHLPP, resulting in increased cell proliferation and resistance to gemcitabine (Figs 7B and 8). Although ERRFI1 binds to both EGFR and AKT, ERRFI1 mainly bound to EGFR upon EGF activation in EGFR-high cells as a tumor suppressor (Figs 3A and Fig EV5A). While in EGFR- low cancer cells, ERRFI1 bound to AKT, but not EGFR regardless of EGF stimulus (Figs 3A and Fig EV5A), therefore, ERRFI1 poses minimum effect on EGFR in EGFR-low cells. This could explain that EGFR signaling is not elevated in EGFR-low cells after knocking down ERRFI1. We have added more discussion on Page 16.

2nd Editorial Decision 20 October 2017

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the referees that were asked to re-evaluate your study (you will find enclosed below). Referee #3 was not able to look at the revised manuscript, but after going through your point-by-point response, I consider his/her points as adequately addressed. As you will see, the other two referees now support the publication of your manuscript in EMBO reports.

Before we can proceed with formal acceptance, I have the following editorial requests that a need to be addressed:

Please provide the three EV datasets and the two EV tables with legends. Please add the legend as a tab in the excel files.

Thank you for providing the source data for the Western blots in Fig. 9. It looks strange to provide this only for 1 figure, and as all the other Western blots have been significantly cropped and

sometimes show strong differences in terms of contrast band size and brightness, we would require the publication of all the original Western blot source data, with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. Please submit scans of the entire blots for all the Western blot panels, including size markers, and label the scans with figure and panel number, and send one PDF file per figure.

Finally, could you please provide a higher quality version of the synopsis figure (in jpeg or tiff format with the exact width of 550 pixels and a height of about 400 pixels)? The version you sent contains compression artefacts, distortions, stripes and the elements loo rather fuzzy.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The authors have adequately addressed my comments from the last review. Additional data generated from breast cancer organoids strengthened the study. I agree that the detailed mechanism underlying ERRFI1-mediated differential regulation of EGFR/Akt signaling can be determined in future studies.

Referee #2:

The authors addressed all questions to my full satisfaction.

Revision - authors' response 12 December 2017

We have addressed the source data as indicated below.
We also renamed the source data for Fig EV4 and Fig EV5 as "Source data for Figure EV4" and "Source data for Figure EV5", and changed the labeling of the panels in these files accordingly.
The revised source data files have been uploaded and submitted to EMBO Reports. Thank you for working with us during the revision process.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Junmei Cairns
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-44767V2

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- 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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Cell-based sample analysis was performed in triplicate for each condition.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	Page 24
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	Page 24
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The data met the assumption of the tests.
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
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http://ClinicalTrials.gov
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http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

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http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

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http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Page 19
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Page 19

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Page 24
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Page 24
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Page 24
12. 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.	Page 24
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Page 24
16. 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.	Page 24
17. 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.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Page 23
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A