EMBO reports

A variable undecad repeat domain in cavin1 regulates caveola formation and stability

Vikas A. Tillu, Ye-Wheen Lim, Oleksiy Kovtun, Sergey Mureev, Charles Ferguson, Michele Bastiani, Kerrie-Ann McMahon, Harriet P. Lo, Thomas E. Hall, Kirill Alexandrov, Brett M. Collins and Robert G. Parton

Review timeline:

Submission date:	10 January 18
Editorial Decision:	21 February 18
Revision received:	29 April 18
Editorial Decision:	28 May 18
Revision received:	5 June 18
Accepted:	14 June 18

Editor: Martina Rembold

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 21 February 18

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, all referees also have a number of suggestions for how the study should be strengthened and ask for further clarification of certain results and experimental strategy, and I think that all of them should be addressed. Referee 3 suggests to test the relevance of the five UC1 repeats in zebrafish cavin1 to mechanical stress resistance to further strengthen the correlation between the number of repeats and caveolar stability. Referee 3 also suggests to remove the data on DR1/3 and cavin1 oligomerization shown in Figure 6 and 7 since these data are either not convincing enough or do not add much to the paper. Upon further discussion with the referees, also referee 1 and 2 agreed with this notion. These figures could thus either be saved for a future study or moved to the supplement to strengthen the focus on the main findings regarding the HR2 domain and the UC1 motif.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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.

You can submit the revision either as a Scientific Report or as a Research Article. A manuscript that contains up to 5 main figures and 5 Expanded View figures will be published as Scientific Report with a combined "Results and Discussion" section. If the revision leads to a manuscript with more than 5 main figures it will be published as a Research Article. In this case the Results and Discussion section can stay as it is now. In either case, all materials and methods should be included in the main manuscript file.

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 with page numbers, 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.

Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied. Please also include scale bars in all microscopy images.

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.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
(In order to avoid delays later in the publication process please check our figure guidelines before preparing the figures for your manuscript:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf)
- a separate PDF file of any Supplementary information (in its final format)
- all corresponding authors are required to provide an ORCID ID for their name. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (http://embor.embopress.org/authorguide).

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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
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Referee #1:

In this paper, Tillu and the colleagues showed that the unique repeated eleven residue sequence (UC1) in the HR2 domain of cavin1 plays essential roles in caveolar distribution and phosphatidylserine binding of cavin1. They also demonstrated that the UC1 domain is important for caveolar stability under mechanical stress using a zebrafish model system. The important role of the N and C terminal disordered regions (DR1 and DR3) for cavin1 oligomerization was also addressed.

This is a well-executed study and clearly showed the importance of the UC1 domain for cavin1 functionalities. Thus, I think this paper is worth publishing in EMBO Reports. My questions and comments are as below:

1. In Figure 1, GFP-cavin1 mutant truncated at the 250th amino acid showed binding to CAV1 and generated caveolae in PC3 cells, although the mutant contains just one repeat of the UC1 domain. Does it indicate that the UC1 domain does not need to form the structure as predicted in Figure 2D to execute its molecular functions? Somewhat in a similar vein, I wonder whether the difference in the number of UC1 domain repeats among different species is reflected in any difference in the functionality of cavin1.
2. In Figure 6A, a cavin1 mutant lacking DR1 alone and the other one lacking both DR1 and DR3 were compared with the full-length cavin1 and it was concluded that at least some cavin1 molecules containing DR1 and DR3 domains must be present for caveola formation. The experiment, however, did not address whether DR3 is necessary. Please clarify this point.
3. I agree with the contention that multiple low affinity interactions between cavin1 and the membrane cooperate to generate the caveola structure. That being said, it is not likely that the binding of HR1 domain to PI(4,5)P2 and the binding of HR2 domain to PS are simply additive. I hope the authors will discuss possible functional difference between these two interactions.

Referee #2:

Tillu, Collins, Parton and colleagues report the identification and characterisation of a variable repeat (UC1) within the HR2 domain that mediates the targeting of cavin1 and thus local membrane remodelling and caveolae formation.

The study was a careful and systematic domain-function analysis of the UC1 domain.
The use of chimera, truncation mutants in several cell biological, microscopic, biochemical and in vivo assays provided multiple lines of evidence supporting the conclusions. The study was very well designed, executed and interpreted and I recommend publication.
I have only a couple of minor comments that the authors may want to clarify:

- What was the rationale for testing the 6KR/ED mutant in A431 instead of PC3 cells (Fig. 3B)? Its normal recruitment to caveolae is in apparent contradiction with its clear defect in PS binding in the liposome binding assay (Fig. 3C). The presence of endogenous cavin1 in A431 cells might compensate for a decrease in membrane binding of the mutant by recruiting it to caveolae (upon heteromerisation) and thus masking the phenotype.

- What could be the structural basis for the preferential binding to phosphatidylserine by the UC1 domain? Other alpha-helices based domains having surface-exposed basic residues (e.g. BAR domains) do not show such discrimination between Pi(4,5)P2 and PS. Could it be because the basic ridges along the surface of the UC1 domains could only fit small negatively-charged headgroups?

Referee #3:

Caveolae are membrane invaginations whose coat proteins interact with the cavin adaptor proteins to form caveolae. Only cavin1 is required for caveolae formation and this paper identifies an eleven-amino acid repeat sequence that is specific to cavin1 and required for caveolae formation and binds to phosphatidyl serine (PS). This is a very interesting story in that it links PS binding domain of cavin1 to caveolae formation and to the caveolae role in mechanical stress. There are some issues that need to be addressed and the manuscript is quite poorly constructed and at times diffuse and should be revised to specifically focus on the role of the undecad repeat.

Comments:
1. Where does the "caveolae localization" column in fig 1 A come from. Is it based on the cavin1-Cav1 PLA analysis in C or EM. This should be clarified and if the former referred to as caveolin association and not caveolae localization.
2. The role of the undecads in caveolae disassembly to mechanical stress is interesting. The specific role of the repeats in the resistance of Zebrafish cavin 1b with 5 repeats should be confirmed by progressive deletion of the repeats. As it stands it is a correlation albeit interesting one. A DUC1 zcavin is used in the zebrafish notochord experiments but a key question is whether loss of the UC1 repeats prevents cavin association with caveolae and even caveolae formation. If so the lack of protective activity of this cavin mutant is not surprising. This should be tested by PLA, as in 3D, and I strongly recommend incorporating the progressive loss of the 5 UC1 repeats into these experiments.
3. The data showing increased tubulation of UC1 containing cavin2 is interesting but the statement on page 9 that the UC1 domain contributes to shape caveolae membrane curvature us not supported by the data - including that shown in Fig S2b.
4. The data in Figures 6 and 7 are less than complete or compelling and do not necessarily add to the paper. Their limited importance to the paper is highlighted by the absence of any reference to this data in the title or abstract. As this is an EMBO Report I recommend that they be deleted.
5. There is a substantial amount of supplemental data and it is not clear if it is necessary and I recommend that the authors review the data and ensure that it is correctly cited and necessary to the manuscript as a whole. In particular, there are two figures of GFP-nanobody blots with references to Figure 2G - that doesnt exist - and 4C that is not related.

The nomenclature for the constructs in the graphs is confusing and should be simplified (3D, 4C, 7A). In 4C the graph should be organized by treatment.

1st Revision - authors' response 29 April 18

Referee #1:
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*We apologise for the confusion. The cavin1 mutant (45-250) is able to generate caveolae in the PC3 cell line only due to the presence of wild-type cavin1-mcherry with which it is able to hetero-oligomerise with (now **Figure EV1B**).*

1b. Somewhat in a similar vein, I wonder whether the difference in the number of UC1 domain repeats among different species is reflected in any difference in the functionality of cavin1.

We thank Referee #1 for this suggestion. We have now performed additional experiments (also suggested by Referee #3) to study the effect of systematically decreasing the number of undecad repeats in the UC1 domain of zebrafish cavin1b. As shown in new ***Figures 4C and 4D*** *we see a clear trend where sequentially reducing the number of UC1 repeats results in a reduced stability of cavolae when challenged by membrane stretching induced by osmotic stress. This supports the notion that the number of UC1 repeats can directly influence the functionality of cavin1.*

2. In Figure 6A, a cavin1 mutant lacking DR1 alone and the other one lacking both DR1 and DR3 were compared with the full-length cavin1 and it was concluded that at least some cavin1 molecules containing DR1 and DR3 domains must be present for caveola formation. The experiment, however, did not address whether DR3 is necessary. Please clarify this point.

At the request of reviewers and the editor, we have now removed figure 6 and 7 related to the disordered regions (DR domains) of the cavin proteins. Our revised manuscript now focuses on the importance and function of the undecad domain as suggested.

3. I agree with the contention that multiple low affinity interactions between cavin1 and the membrane cooperate to generate the caveola structure. That being said, it is not likely that the binding of HR1 domain to PI(4,5)P2 and the binding of HR2 domain to PS are simply additive. I hope the authors will discuss possible functional difference between these two interactions.

We have now included a brief discussion of the potential functional implications of these lipid interactions on page 9.

Referee #2:

Tillu, Collins, Parton and colleagues report the identification and characterisation of a variable repeat (UC1) within the HR2 domain that mediates the targeting of cavin1 and thus local membrane remodelling and caveolae formation. The study was a careful and systematic domain-function analysis of the UC1 domain. The use of chimera, truncation mutants in several cell biological, microscopic, biochemical and in vivo assays provided multiple lines of evidence supporting the conclusions. The study was very well designed, executed and interpreted and I recommend publication. I have only a couple of minor comments that the authors may want to clarify:

1. What was the rationale for testing the 6KR/ED mutant in A431 instead of PC3 cells (Fig. 3B)? Its normal recruitment to caveolae is in apparent contradiction with its clear defect in PS binding in the liposome binding assay (Fig. 3C). The presence of endogenous cavin1 in A431 cells might compensate for a decrease in membrane binding of the mutant by recruiting it to caveolae (upon heteromerisation) and thus masking the phenotype.

*We have now performed additional experiments in PC3 cells to test the ability of the 6KR/ED cavin1 mutant to induce caveola puncta formation (****Figure EV5A****). The cavin1 6KR/ED mutant shows a mild reduction in caveola puncta formation, suggesting that multiple low affinity co-operative membrane interactions from HR1 and UC1 domain are required for efficient caveola formation. For consistency with other experiments we used A431 cells in caveola stability studies (now* ***Figure 4A,B,C****) to analyze the impact of the mutation on caveolae in the presence of endogenous cavin1 and other caveolar coat proteins, and see a clear reduction in caveola stability. Based on the results using PC3 cells shown in* ***Figure EV5A****, the mutant already shows a reduced baseline of caveola puncta formation prior to osmotic stretching, and although we have not performed the experiments we agree with Referee #3 that a stronger phenotype in osmotic stretching assays would likely be observed in PC3 cells.*

2. What could be the structural basis for the preferential binding to phosphatidylserine by the UC1 domain? Other alpha-helices based domains having surface-exposed basic residues (e.g. BAR domains) do not show such discrimination between PI(4,5)P2 and PS. Could it be because the basic

ridges along the surface of the UC1 domains could only fit small negatively-charged headgroups?

Because we don't yet understand precisely how this preference arises, we decided not to speculate about the molecular basis for PS-binding preference in the manuscript. (It is also important to note that this is a 'preference' and not a strict specificity, as sufficiently high (non-physiological) concentrations of either negatively charged PS or PI(4,5)P_2 can compensate for lack of the other lipid.) As Referee #2 suggests, it could be due to the specific alignment of the positively charged residues on the surface of the UC1 domain. We also speculate that the interactions with adjacent Cavin1 trimers assembled together on the membrane could lead to the formation of additional binding sites contributing to PS sensitivity. However, further high-resolution structural studies will be required to determine the mechanisms involved.

Referee #3:

Caveolae are membrane invaginations whose coat proteins interact with the cavin adaptor proteins to form caveolae. Only cavin1 is required for caveolae formation and this paper identifies an eleven-amino acid repeat sequence that is specific to cavin1 and required for caveolae formation and binds to phosphatidyl serine (PS). This is a very interesting story in that it links PS binding domain of cavin1 to caveolae formation and to the caveolae role in mechanical stress. There are some issues that need to be addressed and the manuscript is quite poorly constructed and at times diffuse and should be revised to specifically focus on the role of the undecad repeat.

Comments:
1. Where does the "caveolae localization" column in fig 1 A come from. Is it based on the cavin1-Cav1 PLA analysis in C or EM. This should be clarified and if the former referred to as caveolin association and not caveolae localization.

*The wording in **Figure 1A** is amended appropriately in the revised manuscript.*

2. The role of the undecads in caveolae disassembly to mechanical stress is interesting. The specific role of the repeats in the resistance of Zebrafish cavin 1b with 5 repeats should be confirmed by progressive deletion of the repeats. As it stands it is a correlation albeit interesting one. A DUC1 zcavin is used in the zebrafish notochord experiments but a key question is whether loss of the UC1 repeats prevents cavin association with caveolae and even caveolae formation. If so the lack of protective activity of this cavin mutant is not surprising. This should be tested by PLA, as in 3D, and I strongly recommend incorporating the progressive loss of the 5 UC1 repeats into these experiments.

*As noted by the reviewer the complete deletion of the UC1 domain does indeed result in a reduced caveola-association and cavolae number (**Figure 3B**). The goal of the experiments in cavin1b-/- zebrafish where rescue experiments are performed was to confirm that this deletion of the UC1 domain resulted in a functional deficit in vivo (now **Figures 3C,D,E**). Performing further truncation experiments in the zebrafish model would be excessively time-consuming and would not provide the precise quantitative assessment of caveolar stability that we can achieve in cultured cells. However, as suggested by Referee #3 (and Referee #1) we have now extended the analysis of the UC1 domain in A431 cells using the zcavinsb protein as a model (with five UC1 undecad repeats versus the mouse protein which has two) (**Figures. 4C,D**). Firstly we show that compared to the mouse cavin1 protein the zcavin1b protein provides greater resistance to osmotic stretching as measured by PLA assay. Secondly we truncated each undecad repeat individual and now show the effect of progressively deleting each of the UC1 repeats. This establishes a clear trend whereby successive deletions result in decreased stability in the model cell system but importantly has minimal effect on cavin1 association with caveolae. We believe that these new experiments considerably strengthen the conclusions of the manuscript.*

3. The data showing increased tubulation of UC1 containing cavin2 is interesting but the statement on page 9 that the UC1 domain contributes to shape caveolae membrane curvature us not supported by the data - including that shown in Fig S2b.

*We have rephrased this section to better reflect the results observed in **Figure 5**.*

4. The data in Figures 6 and 7 are less than complete or compelling and do not necessarily add to the paper. Their limited importance to the paper is highlighted by the absence of any reference to this data in the title or abstract. As this is an EMBO Report I recommend that they be deleted.

As requested by Referee #3 and the editor after conversation with the other referees we have now removed Figure 6 and 7. The manuscript is now solely focused on the role of the UC1 domain undecad repeats in cavin1 function.

5. There is a substantial amount of supplemental data and it is not clear if it is necessary and I recommend that the authors review the data and ensure that it is correctly cited and necessary to the manuscript as a whole. In particular, there are two figures of GFP-nanobody blots with references to Figure 2G - that doesnt exist - and 4C that is not related.

We have amended the text and figures to streamline the manuscript as much as possible in the revised version, and corrected the errors in figure citations.

The nomenclature for the constructs in the graphs is confusing and should be simplified (3D, 4C, 7A). In 4C the graph should be organized by treatment.

This has been changed in the revised manuscript.

We believe that the revisions have considerably strengthened the manuscript and hope that the revised manuscript proves suitable for publication in *EMBO Reports.*

2nd Editorial Decision 28 May 18

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

- Please note that all corresponding authors are required to provide an ORCID ID for their name. Therefore, please provide an ORCID for the co-corresponding author Robert Parton. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (http://embor.embopress.org/authorguide).

- Please specify the contributions of Sergey Mureev and Kirill Alexandrov in the Author Contributions section.

- You refer to Appendix Fig.S1 in the manuscript (page 13, line 393), which is not provided. Should the callout refer to Figure EV1 instead?

- Please reformat the references to match the numbered style of EMBO reports. You can download the respective EndNote file from our Guide to Authors using the following link: https://drive.google.com/file/d/0BxFM9n2lEE5oOHM4d2xEbmpxN2c/view

- Figure 5A: The magnification boxes in the overview image in the right panel are rather thin and might not be well visible at final print size. Also the scale bars in these images are quite thin lines and I suggest making them thicker.
- In general, please review all scale bars in regard of their visibility at final print size (180 mm width). Some of the green scale bars, e.g., in Fig. EV1 are not well visible.

- Our data editors from Wiley have already inspected the Figure legends for completeness and accuracy. Please see their suggested changes in the attached Word file.

- The synopsis image looks very good but I suggest having another look at it at final resolution, i.e., at 550 pixels width. Doing so, you will see that the stippled line is not very well visible and as such it is not obvious on first glance that the bottom images are close-ups of the UC1 domain. The text at the bottom could also be a bit larger. The dimensions of the synopsis image can be up to 400 x 550 pixels.

Once you have made these minor revisions, please use the following link to submit your corrected manuscript:

https://embor.msubmit.net/cgi-bin/main.plex?el=A3Ij4jh1A6CFgL4J6A9ftdV9et2uywvcMEJIKAK0oLAQY

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORTS
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Referee #1:

The authors revised the manuscript adequately. I think the paper is acceptable in the present form.

Referee #2:

The authors addressed all my comments and improved considerably their manuscript.
I do recommend publication.

Referee #3:

The authors have addressed my concerns and this is a very interesting story on a novel Cavin1 domain and its role in caveolae stabilization.

2nd Revision - authors' response 5 June 18

The authors made all suggested changes.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Robert Parton and Brett Collins
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-45775V1

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 $\chi 2$ 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.

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?	All experiments were repeated multiple times idependendly. sample size (n) and number of independent biological replicates (N) for each experiment are mentioned in the figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	All animal experiments were repeated multiple times independently. Sample size (n = number of embryos) and independent biological clutch of zebrafish (N) for each experiment are mentioned in the figure legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not applicable.
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.	No.
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used.
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.	No blinding was done
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done
5. For every figure, are statistical tests justified as appropriate?	Yes,Statistical significance was assessed using one way ANOVA test
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes , Statistical analysis were performed using GraphPad Prism 7.0
Is there an estimate of variation within each group of data?	NA

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http://1degreebio.org
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http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	YES

C- Reagents

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).	Yes, Page 11 Article materials and Methods
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes. A431 and PC3 cell lines were obtained from American Type Culture Collection (ATCC). Cell were tested for mycoplasma contamination routinely.

* 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.	Yes, Description can be found in materials and methods section
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were approved by The University of Queensland Animal Ethics Committee.
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.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	NA
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).	NA
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).	NA
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.	NA

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.	No.