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Prdx4 limits caspase-1 activation and restricts inflammasome-mediated signaling by extracellular vesicles

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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 20th Dec 2018

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

Both referees find the analysis interesting, but they also indicate that further analysis is required. In particular the referees find that further data is needed to support a causal role of Prx4 activity in inflammatory signalling and where it fits in the pathway. Also referee #2 is asking for more insight into how Prx4 and caspase-1 end up in the same vesicles. Both points raised are reasonable. For the point regarding how Prx4 and caspase-1 meet, we don't need the complete mechanism but some additional insight would be good.

Should you be able to address the raised concerns, then I would like to invite you to submit a revised version. I should add that it is EMBO Journal policy to allow only a single major round of revision, and that it is therefore important to resolve the major ones at this stage.

REFEREE REPORTS:

Referee #1:

General summary and opinion about the principle significance of the study, its questions and findings
The significance of this work is contextualized within a widely contentious area concerning the mechanism of IL-1β secretion and its subsequent cell to cell signaling. Specifically, the work aims to support a model wherein Peroxiredoxin-4 (Prdx4) directly regulates Caspase-1 activity within extracellular vesicles (EVs) to control IL-1β maturation. While the submitted work does clearly support a role for Prdx4 in inflammatory signaling using genetics, the biochemical insights provided

are without strong experimental support. One major limitation of the work is a robust demonstration that Prdx4 acts downstream of Caspase-1. Accurately positioning Prdx4 at this step is critical for maintaining its proposed mechanism of action.

Specific major concerns essential to be addressed to support the conclusions:

Many of the experimental data used by Lipinski et al. to support their model rely almost exclusively on NLRP3 mediated Caspase-1 activation. Inflammasome signaling driven by NLRP3 is unique amongst other PRRs owing to its regulation by two distinct signals. The first signal, also known as priming, is responsible for inducing the expression of both NLRP3 and pro-IL-1β, while the second signal triggers NLRP3 inflammasome assembly (eg. ATP and Nigericin). Priming is positively regulated by NFκB signaling, a pathway known to be negatively regulated by Prdx4. The authors do address priming defects as a consequence of Prdx4 deficiency, but they limit their analysis prematurely to the transcriptional level.
It remains possible that Prdx4 may negatively regulate NLRP3 at the protein level. To examine this possibility, the authors are encouraged to demonstrate that Prdx4 does not impinge on NLRP3 protein levels and or stability.
From this point, the study takes a dramatic shift to support a secretory role for Prdx4 in regulating Caspase-1 activity inside extracellular vesicles. The authors begin to support this model first by showing that Prdx4 is secreted into the tissue culture (TC) supernatant upon NLRP3 activation using ATP. Even though the secretion of Prdx4 is shown to require Caspase-1 activation (YVAD blocks Prdx4 secretion), no experimental support is provided to rule out alternative modes of non-vesicular secretion. This is peculiar given the authors cite alternative modes of secretion for IL-1β itself (eg. Gasdermin D pores, passive loss of cellular integrity upon pyroptotic cell-death etc.).
Nonetheless, this observation prompts the investigators to examine the TC-supernatant for evidence of extracellular vesicles (EVs) citing previous studies showing ATP stimulation permits non-conventional vesicle shedding. Strangely, no attempt is made to test whether vesicle shedding is observed in response to other NLRP3 agonists, or by different inflammasome sensors (AIM2 or NLRC4). This is critical for supporting the earlier data showing the phenotype accompanying Prdx4 deletion is independent of inflammasome identity. Notwithstanding, the TEM data used to support EV isolation is of poor quality. What's more, no orthogonal technique is used to unequivocally prove EVs have been isolated (eg. Dynamic Light Scattering). Although CD63 is used as a marker to confirm isolation of EVs, no markers serving as negative controls are included. Mature IL-1β in EV preparations is undetected, which prompts evaluation of their bioactivity in assays downstream of the IL-1β receptor. No measure, however, is taken to control for the relative dose of administered EV preparations.
In the final two figures, Lipinski et al. provide experimental support for a direct interaction between Prdx4 and Caspase-1 using recombinant protein or heterologous expression in 293T cells. Specifically, the association between Prdx4 and Caspase-1 is attributed to disulfide bond exchange. Perhaps this is expected since Prdx4 is known to mediate disulfide bond formation in the endoplasmic reticulum through successive rounds of oxidation-reduction. Thus, disulfide bond exchange is unlikely to display specificity given access to an alternative client protein. Nevertheless, exchange reactions could benefit from inclusion of a negative control. Since the site of exchange is mapped to Caspase-1, corresponding sites on Prdx4 should be reciprocally tested.

Referee #2:

The paper considers the role of Prx4 in the inflammasome-mediated immune response by analysing the effect of Prx4 KO on a variety of measures of response. The data demonstrates a clear phenotype of KO but falls short of a clear understanding of the link between cause and affect. The function of Prx4 is to reduce peroxides. Its absence will inevitably lead to an increase in peroxides generated in the ER. In addition no attempt is made to resolve how Prx4 (an ER protein) and caspase1 (a cytosolic protein) end up in exosomal vesicles. It is actually not clear that they are co-localised in these vesicles as stated by the authors as there is no evidence presented that they are both present in the same vesicle. Without these questions being addressed the paper provides a general observation but does not provide any mechanistic insight into how Prx4 could control the immune response.
There are some very basic inaccuracies in the paper when the authors evaluate the form of Prx4 the interacts with caspase1. They infer a monomer, dimer, decamer transition from studying the banding

patterns by SDSPAGE. It is highly likely that Prx4 remains as a decamer throughout this process. What the authors are actually analysing is the denatured protein and the reduction of disulfides which would covalently link the individual subunits so that they form dimer or decamers under denaturing conditions. There is actually no evidence presented that suggests Prx4 subunits dissociate from a decamer under reducing conditions. For this analysis they would need to determine the oligomeric status under native conditions by for example size exclusion chromatography or sucrose gradient analysis. The specifity they do see between reduced and oxidised Prx4 is most likely due to a disulfide being formed between the resolving thiol in Prx4 and a thiol in caspase1.
In summary, the work is lacking a link between cause and affect, does not demonstrate a link between Prx4 activity and the phenotypes they see and the work should be supported by some mechanistic framework to explain how the proteins actually meet each other.

1st Revision - authors' response 24th May 2019

Referee #2:

The paper considers the role of Prx4 in the inflammasome-mediated immune response by analysing the effect of Prx4 KO on a variety of measures of response. The data demonstrates a clear phenotype of KO but falls short of a clear understanding of the link between cause and affect.
1. The function of Prx4 is to reduce peroxides. Its absence will inevitably lead to an increase in peroxides generated in the ER. In addition no attempt is made to resolve how Prx4 (an ER protein) and caspase1 (a cytosolic protein) end up in exosomal vesicles. It is actually not clear that they are co-localised in these vesicles as stated by the authors as there is no evidence presented that they are both present in the same vesicle. Without these questions being addressed the paper provides a general observation but does not provide any mechanistic insight into how Prx4 could control the immune response.
Response: We would like to thank for this useful comment, which has clearly helped us to improve our manuscript. To elucidate how Prdx4 and caspase-1 end up in extracellular vesicles, we performed several additional experiments. We now show this data combined with previous data in **Figure 5** and have restructured the manuscript accordingly (lines 249-260 and 268-287). We first performed cellular fractionation and observed that Prdx4 is present in the cytosolic compartment, which is in accordance with literature (Jin, Chae et al., 1997, Tavender, Sheppard et al., 2008) and database predictions, also including EV contents (Pathan, Fonseka et al., 2019, UniProt Consortium, 2018). Cytosolic levels increased in response to inflammasome activation (**Figure 5A**). This suggested that a proportion of Prdx4 is contained in the cytosol, where it is thus able to interact with caspase-1. Cytosolic proteins can be sorted and sequestered into multivesicular bodies (MVB) in an ESCRT-complex-dependent manner (Babst et al., 2002, Teis et al., 2008) and released as cargo from MVBs as extracellular vesicles (TheryWitwer et al., 2018, Tkach & Thery, 2016). Thus, we blocked the release of mature extracellular vesicles from MVBs using the inhibitor GW4869 (Trajkovic, Hsu et al., 2008, Kosaka et al., 2010, Mittelbrunn et al., 2011). Indeed, we found that GW4869 significantly lowered Prdx4 as well as caspase-1 secretion (**Figure 5F**). To strengthen the conclusion that that cytosolic Prdx4 together with cytosolic caspase-1 might be sorted into MVBs and released via EVs from the cell, we then rearranged out data, showing that Prdx4 was enriched in CD63+ fractions, a marker for MVBs (Kobayashi, Vischer et al., 2000) (**Figure 5G and H**). Moreover, these fractions were also positive for ASC, pro-caspase-1 and pro-IL-1b. Thus, these results provide evidence that Prdx4 and caspase-1 are co-present in MVBs, suggesting their joint release from inflammasome-activated cells.
We have also performed new experiments to investigate whether Prdx4 and caspase-1 are co-localized within the same vesicle. To this end, we used immunofluorescence microscopy as previously described for EVs (Athman, Wang et al., 2015). Our new set of data is now displayed in **Appendix Figure S6**. We observed a high degree of co-localization for EVs derived from LPS+ATP stimulated BMDMs that were stained for Prdx4 and caspase-1 and counterstained with a lipophilic dye for the EV membrane (text in lines 319-325).
Collectively, these data indicate that distinct EV populations are released in response to NLRP3 inflammasome activation, containing constituents of the inflammasome as well as Prdx4.

2. There are some very basic inaccuracies in the paper when the authors evaluate the form of Prx4 the interacts with caspase1. They infer a monomer, dimer, decamer transition from studying the

banding patterns by SDS-PAGE. It is highly likely that Prx4 remains as a decamer throughout this process. What the authors are actually analysing is the denatured protein and the reduction of disulfides which would covalently link the individual subunits so that they form dimer or decamers under denaturing conditions. There is actually no evidence presented that suggests Prx4 subunits dissociate from a decamer under reducing conditions. For this analysis they would need to determine the oligomeric status under native conditions by for example size exclusion chromatography or sucrose gradient analysis. The specifity they do see between reduced and oxidised Prx4 is most likely due to a disulfide being formed between the resolving thiol in Prx4 and a thiol in caspase1.

Response: We apologize for the obvious inaccuracies in the visualization and text of the previous version, which referred to the oligomeric state of Prdx4. We fear that this might have led to a misunderstanding and we thank the reviewer for pointing this out.

It was never our intention to infer a monomer, dimer, decamer transition of Prdx4. Rather, we agree with the reviewer's argument that Prdx4 presumably remains a decamer throughout the process. In fact, this is supported by the former **Figure 5B** and **C**, now **Figure 4A** and **B**, showing that the majority of Prdx4 is present as a high MW oligomer. We suspect that our former **Figure 5A** was misleading and caused the reviewers scepticism. Our original intention to show this picture of an SDS-PAGE loaded with *in vitro* reduced and non-reduced forms of Prdx4 was to familiarize the reader with the multimeric structure of Prdx4 and to determine the electrophoretic properties of the oligomers and the monomer of Prdx4. We are aware of the fact that this migration pattern is due to denaturing conditions of the SDS-PAGE. We have now removed the picture of the SDS-PAGE and the graphical illustration of Prdx4 and deleted the misleading statement (in the former manuscript lines 245-247). We have carefully rephrased the respective paragraphs and tuned down claims about the exact oligomer structure ("high molecular weight oligomer"), which we only infer from literature to be a decamer.

Our main point, however, is to emphasize our finding (**Figure 4A**) that caspase-1 co-migrates with the Prdx4 oligomer, which was the starting point for the subsequent analysis. We have now inserted additional data including recombinant GAPDH as a control for specificity (please see also response 4 to referee#1). GAPDH was chosen because of its localization in the cytosol and because it contains an active-site cysteine, known to be redox-sensitive (Nakajima et al., 2009). However, co-incubation with Prdx4 had no impact on the distribution and migration patterns of the homotetrameric rGAPDH (**Appendix Figure S5B**), indicating that disulfide bond exchange between caspase-1 and Prdx4 is not necessarily unspecific (lines 208-212) and, indeed, most likely occurs between one thiol of Prdx4 and the free thiol in caspase-1. We also performed native gel electrophoresis and confirmed the co-migration pattern of rCASP-1 and rPRDX4 in the absence of detergents.

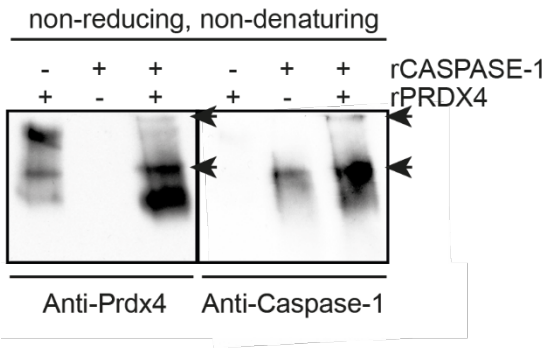

Figure I. Native gel electrophoresis and Western blot analysis of rCASP-1 and rPRDX4 that were incubated either individually or co-incubated as indicated.

However, what we also found is that the presence of caspase-1 alters the SDS-PAGE migration pattern, of Prdx4 upon their co-incubation *in vitro* and ii) co-expression in cells, when analysed under non-reducing and denaturing conditions (**Figure 4A and B**) as well as non-reducing + non-denaturing conditions (**Figure I, not included in manuscript**). At this point, we can only speculate that the appearance of this lower molecular weight band of Prdx4 at the expected size of the reduced dimer, are indeed dimers. Although a redox-dependent Prdx4 decamer-to-dimer transitions has been demonstrated before (Tavender & Bulleid, 2010, Tavender, Springate et al., 2010), we refrain from claiming that these bands are indeed dimers that were released from high-molecular weight complexes, as much more detailed experiments would be needed to show the exact structure and conditions of the transition. As in fact this point is less relevant for the biological interpretation of

our main findings, we have not included Figure I and carefully tuned down our statements (lines 346-351 in the former manuscript).
In summary, the work is lacking a link between cause and affect, does not demonstrate a link between Prx4 activity and the phenotypes they see and the work should be supported by some mechanistic framework to explain how the proteins actually meet each other.

We appreciate the reviewer´s criticism, which has led to a series of rewarding experiments for deeper mechanistic exploration. In our view, we now present a compelling set of novel data and hope to meet the reviewer's expectations.

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2nd Editorial Decision 19th Jun 2019

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by the two referees. As you can see below, both referees appreciate the introduced changes and support publication here.

Referee #2 has one remaining issue that should be sorted out and that is concerning the sub-cellular location of Prx4. Can you please carefully check the cited references and make sure that you describe the literature is a good manner. Also, please make sure that you describe how you did the experiments in Figure 5A.

When you submit the revised version would you also take care of the following editorial points.

REFEREE REPORTS:

Referee #1:

All concerns adequately addressed.

Referee #2:

The manuscript is much improved but I am very frustrated by a lack of clarity in the experiments aimed at resolving the sub-cellular location of Prx4. I have looked carefully at the past literature cited by the authors but see no definitive evidence that the protein is located in the cytosol. The first paper Jin et al 1997 fig. 5B notes localisation in what they call a cytosolic fraction but this is essentially everything that is not in a nuclear fraction and so could contain ER proteins. The second paper, Tavender et al, 2008 shows by immunofluorescence that the protein is localised to the ER (Fig. 2). The way these papers are refered to is therefore quite misleading (lines 253-256). The authors own data (Fig. 5A) shows Prx4 in both a cytosolic and an insoluble fraction. However, I failed to find anywhere in their figure legend or the methods how these fractions were generated which is obviously important information. They have a section in the methods on subcellular fractionation but that specifically refers to the density gradient analysis. Hence I would like to see this point cleared up so that the reader can make up their own minds as to the validity of the claims made.

2nd Revision - authors' response 5th Aug 2019

Reply to comments @ EMBOJ-2018-101266R "Prdx4 limits caspase-1 activation and restricts inflammasome-mediated signaling by extracellular vesicles" by Lipinski et al.

"Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by the two referees. As you can see below, both referees appreciate the introduced changes and support publication here.

Referee #2 has one remaining issue that should be sorted out and that is concerning the sub-cellular location of Prx4. Can you please carefully check the cited references and make sure that you describe the literature is a good manner. Also, please make sure that you describe how you did the experiments in Figure 5A. "

We took care of all editorial comments. All changes in the manuscript have been highlighted in blue.
When preparing the source data files, we found two unintentionally inserted errors: i) The blot in Figure 3C was lacking a horizontal line indicating splicing of the blot. The original source data file showing the complete including the irrelevant lanes has been uploaded. ii) In Figure 5A, the molecular weight marker for Prdx4 indicated 120 kDa instead of 25 kDa. We apologize for these inaccuracies and have now corrected these mistakes.

Referee #1:

All concerns adequately addressed.

Referee #2:

The manuscript is much improved but I am very frustrated by a lack of clarity in the experiments aimed at resolving the sub-cellular location of Prx4. I have looked carefully at the past literature cited by the authors but see no definitive evidence that the protein is located in the cytosol. The first paper Jin et al 1997 fig. 5B notes localisation in what they call a cytosolic fraction but this is essentially everything that is not in a nuclear fraction and so could contain ER proteins. The second paper, Tavender et al, 2008 shows by immunofluorescence that the protein is localised to the ER (Fig. 2). The way these papers are refered to is therefore quite misleading (lines 253-256). The authors own data (Fig. 5A) shows Prx4 in both a cytosolic and an insoluble fraction. However, I failed to find anywhere in their figure legend or the methods how these fractions were generated which is obviously important information. They have a section in the methods on subcellular fractionation but that specifically refers to the density gradient analysis. Hence I would like to see this point cleared up so that the reader can make up their own minds as to the validity of the claims made.
Reply:

We appreciate the reviewer´s feedback and apologize for the lack of clarity.

We have addressed the comment in several ways to increase transparency and to allow a fair assessment of the observations by the reader. (1) We have inserted the detailed experimental procedure of the cell fractionation in the material and methods part (lines 628-635). To obtain the cytosolic fraction, we treated the cells with a hypotonic lysis buffer cells followed by mechanical disruption and 2 centrifugation steps (10 min at 1,000×g and 30 min at 20,000×g). The pellet of the second step was used as "insoluble fraction". This is a slight modification of the protocol by Song et al., Proteomics 2006. Of course, we cannot completely rule out that smaller quantities of selected ER proteins may still be present in this fraction. (2) We thus have carefully rephrased the section on past literature and cite two systematic proteomics/immunofluorescence studies, which are suggestive of a pool of cytosolic Prdx4 (Itzhak et al., 2016 Elife and Thul et al., Science 2017). We, however, now also clearly state that the majority of Prdx4 can be found in the ER, which we never put into question. (3) We have added another sentence stating the complexity of the compartmentalization of

the inflammasome apparatus and pro-IL1b at the end of the respective section to further tune down the claims.

3rd Editorial Decision 21st Aug 2019

Thanks for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a look at everything and I am pleased to accept the manuscript for publication here.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Philip Rosenstiel
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2018-101266

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?	We chose the sample size for in vivo and in vitro experiments based on previous experiments. The number of independent experiments and technical replicates is indicated in the respective figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The envisaged number of animals and group size of n = 10 is for statistical hedging with 1. Probability of error 1. Type: 0.05 (5%) 2. Desired statistical power of 0.8 3. Variance or effect strength (indicating the parameter used, the effect intensity according to Cohen) d = 1.4 (effect intensity according to Cohen).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No aminals or samples were excluded.
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.	We selected male mice that were randomly distributed in different groups.
For animal studies, include a statement about randomization even if no randomization was used.	We did not use a specific method for randomization
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.	We divided anminals randomly into different groups. We assured that measurements (e.g. weight measurements) were preformed according to the four eyes principle.
4.b. For animal studies, include a statement about blinding even if no blinding was done	We used blinded genotypes for the initial experiment investigating the genotype-dependent effect in response to the LPS-induced septic shock (Figure 1A). For subsequent experimens, no blinding was used.
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.	Yes, we performed all analyses using prism software. We described the statistical methods in the the figure legends and the material and methods section.
Is there an estimate of variation within each group of data?	Yes, we presented every data as mean +/- standard deviation.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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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).	We specified all antibodies used in the material and methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK cells were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) and routinely tested for mycoplasma contamination twice a year.

* 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.	We reported all information in the material 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 experiments were carried out according to the German Animal Protection Law and in accordance with the guidelines for Animal Care of the University of Kiel Vote No.: V242-7224.121-33 (99-7/13) and (156-11/13).
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.	We confirm compliance.

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.