AIFM1 is a component of the mitochondrial disulfide relay that drives complex I assembly through efficient import of NDUFS5

Silja Salscheider, Sarah Gerlich, Alfredo Cabrera-Orefice, Esra Peker, Robin Rothemann, Lena Murschall, Yannik Finger, Karolina Szczepanowska, Zeinab Ahmadi, Sergio Guerrero-Castillo, Alican Erdogan, Mark Becker, Muna Ali, Markus Habich, Carmelina Petrungaro, Nele Burdina, Guenter Schwarz, Merlin Klussmann, Ines Neundorf, David Stroud, Michael Ryan, Aleksandra Trifunovic, Ulrich Brandt, and Jan Riemer

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| Event                          | Date      |
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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)
Thank you again for submitting your manuscript EMBOJ-2022-110784 to The EMBO Journal. Please also excuse the delay in communicating a decision to you, which was due to two repeatedly delayed referee reports, as previously mentioned. Despite having been in contact with the referees, we still have not received one of the reports, and in the interest of time, we have decided to nonetheless proceed and make a decision based on the two reports that we have (copied below for your information). Given these comments, we would now invite you to prepare and submit a revised version of the manuscript.

As you will see, both referees acknowledge the interest of the findings for the field, but also have several concerns that should be resolved in the revised version. Specifically, please revise the text and add experimental data where applicable to respond to all of referee #1’s comments. In addition, the discussion should be expanded to address referee #2’s general concern regarding the mechanism how the AIFM1-MIA40 complex affects the import of specific substrates in more detail. Please also carefully consider all of the specific points referee #2 raises and revise the text or figures as appropriate.

Please note that it is our policy to allow only a single round of major revision. Acceptance depends on a positive outcome of a second round of review and therefore on the completeness of your responses included in the next, final version of the manuscript. It is thus important to clarify any questions and concerns at this stage and I encourage you to review the referees’ comments and to contact me to discuss specific points or a preliminary revision plan in case there are any uncertainties regarding the revision.

Thank you for the opportunity to consider your work for publication. I look forward to receiving your revised manuscript.
Referee #1:

The authors present here an analysis of the role of AIFM1 that has been described so far to play a role in import of MIA40, the central component of the IMS import machinery. The MIA import machinery traps incoming precursor proteins via oxidative protein folding. It is found here now that AIFM1 forms a stable complex with MIA40. Surprisingly, MIA40 levels do not change when AIFM1 is absent but seems to have less binding affinities to its substrates and consequently import is reduced. In an elegant combination of global profiling approaches including in depth complexome profiling and functional assays the authors now identified with the Complex I subunit NDUFS5 a particularly strong affected substrate of this pathway. Non-imported NDUFS5 precursor is then subjected to proteasomal degradation in the cytosol. AIFM1 function therefore goes beyond its previous role in MIA40 import but appears to be required for stable precursor interaction with MIA40.

This study is well conducted and provides novel general insights into the mitochondrial protein import field. It is in my opinion also well suited for a readership interested in general molecular mechanisms in life sciences. I can therefore in principle recommend publication in EMBO Journal.

I have a few issues that should be clarified before publication:
- the manuscript is not really easy to read. E.g. it takes quite a while to understand Figs. 2A S4 and S5. It would be good if the authors could try to focus and combine some of their results to an more easier understandable way and try to make the text a bit smoother for reading
- Can the complexome data be provided on a public server? I think that many mitochondrial researchers might be interested to search these data.
- Figure 1 D: reexpression of AIFM1 in AIFM KO seems to lead to much higher MIA40 levels as in WT (and compared to the affected substrates). What could be the reason for this? In turn, would the import of NDUFS5 be back to WT level (see Fig 3A, here only the KO was tested against WT and not the reexpression of AIFM1). Or would the import be even stronger than WT?
- Fig 3A is there a reason that only NDUFS5 and NDUFB7 imports were tested? How would other affected substrates import?
- is there an in vitro model available to test the proposed binding dependency of NDUFS5 with MIA40 in the presence or absence of AIFM1? Is it really the binding with AIFM1 that modulates MIA40 substrate binding capacity or could the absence of AIFM1 allows modification of ‘orphan MIA40’ (e.g. phosphorylation etc...)?
- it is interesting that the AIFM1-MIA40 complex seems to be strongly required for complex I but rather not for complexes III and IV. Did the authors import the C IV assembly factors COA5, COA6 or CMC2 in AIFM1 mitochondria? As these ones also appear to be dysregulated here (Fig. 1E). Yeast Coa6 e.g. is Mia40-dependent (Vögtle 2012 MPC).
Referee #2:

The mitochondrial MIA pathway with MIA40/CHCHD4 mediates the import and oxidative folding of many intermembrane space (IMS) proteins. In the present study, Salscheider et al. report that AIFM1 forms a stable complex with MIA40, which could be important for the import of not only MIA40 itself but also some MIA-pathway substrates including NDUFS5, which facilitates assembly of complex I in the mitochondrial inner membrane. This finding is of high interest because it suggests the existence of previously overlooked aspects, other than the protein level and redox state, of MIA40 that are important for the operation of the MIA-pathway import. The experiments were carefully designed, the employed experimental techniques are very high, and interpretation of the obtained results is appropriate. However, it is unclear how the AIFM1-MIA40 complex, which requires AIFM1 dimerization, affects the import of some MIA pathway substrates, and there was no attempt to address this question experimentally. Localization of MIA40 to the inner membrane by AIFM1 could decrease the efficiency of MIA40 receiving its substrates from the TOM complex, but this is contrary to the results shown here. Since this work is rich in the data on the roles of NDUFS5 in the complex I assembly, the manuscript could be altered to the one fitting better for the EMBO J; the title and direction could be changed to those addressing the above line of questions rather than the still-elusive new role of the AIFM1-MIA40 complex in its import of some substrate proteins.

Specific points:
- Page 3, lines 5-6: AIFM1-mediated import of MIA40/CHCHD4 relies on the N-terminal region in MIA40/CHCHD4 and the presence of the dimeric form of AIFM1 (Hangen et al., 2015). It looks that the dimerization of AIFM1 is not described in Hangen et al., 2015.
- Fig. S1A: It is difficult to see the shapes of mitochondria due to poor resolution.
- Fig. S1B: The figure showed that the shift of MIA40 to the oxidized state was delayed in AIFM1 KO cells, but in the main text, it is described as "almost unchanged".
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- Fig. 4D: Apparent MWs of the complexes should be shown.
- Fig. 5E: Why does the ratio of AIFM1 in the complex with MIA40 and in the free form differ among different tissues? Do the expression levels of NDUFS5 and levels of complex I differ among different tissues?
Point-by-Point response to the referees

We thank the referees for their thorough assessment of our work. We responded to all points of both referees and provide additional experimental data.

Referee #1:

The authors present here an analysis of the role of AIFM1 that has been described so far to play a role in import of MIA40, the central component of the IMS import machinery. The MIA import machinery traps incoming precursor proteins via oxidative protein folding. It is found here now that AIFM1 forms a stable complex with MIA40. Surprisingly, MIA40 levels do not change when AIFM1 is absent but seems to has less binding affinities to its substrates and consequently import is reduced. In an elegant combination of global profiling approaches including in depth complexome profiling and functional assays the authors now identified with the Complex I subunit NDUFS5 a particularly strong affected substrate of this pathway. Non-imported NDUFS5 precursor is then subjected to proteasomal degradation in the cytosol. AIFM1 function therefore goes beyond its previous role in MIA40 import but appears to be required for stable precursor interaction with MIA40.

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We thank this referee for the positive assessment of our work.

I have a few issues that should be clarified before publication:

- the manuscript is not really easy to read. E.g. it takes quite a while to understand Figs. 2A, S4 and S5. It would be good if the authors could try to focus and combine some of their results to an more easier understandable way and try to make the text a bit smoother for reading

We appreciate this remark and have simplified Figure 2A summarizing the overall assembly pathway and the observed abundance changes of the different intermediates. Moreover, we have thoroughly rewritten the sections describing the results obtained by complexome profiling to make the text easier to read and better guide the reader through the data. We would like to note however that the wealth of information provided by this experimental approach and the complicated Complex I assembly pathway place limits to the extent the description could be simplified.

- Can the complexome data be provided on a public server? I think that many mitochondrial researchers might be interested to search these data.

Already for the previous manuscript version we uploaded the data to a public server and we also provided a data availability statement that reads as follows: “The datasets of the complexome profiling experiments in the current study are available in the CEDAR database (https://www3.cmbi.umcn.nl/cedar/browse/) with the accession code CRX34. Further datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.”
- Figure 1 D: reexpression of AIFM1 in AIFM KO seems to lead to much higher MIA40 levels as in WT (and compared to the affected substrates). What could be the reason for this?

In turn, would the import of NDUFS5 be back to WT level (see Fig 3A, here only the KO was tested against WT and not the reexpression of AIFM1). Or would the import be even stronger that WT?

Indeed, reexpression of AIFM1 in AIFM1 knockout cells leads in Figure 1D to increased MIA40 levels compared to wildtype cells. AIFM1 is reexpressed in the knockout cells using an inducible promotor. We think that differences in the duration of AIFM1 expression explain differences in MIA40 levels. In Figure 1D expression of AIFM1 was induced for 1 day, while in Figure S2A, AIFM1 expression was induced for 3 days (here MIA40 levels are not increased compared to WT). In our hands, expression of AIFM1 for the shorter time often resulted in increases of MIA40 levels that “nomalized” again after 3 days. Since AIFM1 is important for rapid MIA40 import kinetics, its expression might on the short-term increase import and thus MIA40 levels. In the long term, MIA40 levels might be balanced out by e.g. an increase in its degradation. We now indicate the different times of AIFM1 reexpression in the figure legends.

Reexpression of AIFM1 recovers NDUFS5 levels. This can be nicely seen by the complete recovery of complex I levels in our complexome profiling experiments but also in Figure S10. Here AIFM1 was reintroduced and expressed for 1 day and as can be seen MIA40 levels are slightly increased, while NDUFS5 levels are similar to wild type levels.

- Fig 3A is there a reason that only NDUFS5 and NDUFB7 imports were tested? How would other affected substrates import?

We focussed on the characterization of NDUFS5 because it was the MIA40-dependent complex I subunit that was most affected. NDUFB7 served as a control due to its structural similarity in the twin-CXnC region to NDUFS5. To address the reviewer’s concern, we now also performed import experiments with NDUFA8 and NDUFB10 into mitochondria isolated from HEK293 cells (either wildtype or AFM1 knockout; see below, for CMC2 and COA6 see the respective answer). Import was like for NDUFB7 only very mildly affected. We decided to present this data in this response to the referee’s section only, as the manuscript is already quite data-rich.

- is there an in vitro model available to test the proposed binding dependency of NDUFS5 with MIA40 in the presence or absence of AIFM1? Is it really the binding with AIFM1 that modulates MIA40 substrate binding capacity or could the absence of AIFM1 allows modification of ‘orphan MIA40’ (e.g. phosphorylation etc.....)?

We currently establish in vitro reconstitution systems of MIA40 and AIFM1. This system will allow to answer detailed mechanistic questions in the future. This however is a massive undertaking and in our view beyond the scope of the present study.
Our model of MIA40-AIFM1 action and its impact on substrate import mainly stems from the loss of the transient MIA40-NDUFS5 interaction in the AIFM1 knockout cells during import and the fact that MIA40 substrates are affected quite differently by the loss of AIFM1.

We have unpublished data pointing to posttranslational modifications on MIA40. Whether these modifications influence interaction with AIFM1, substrate import or rather the import and maturation of MIA40 still needs to be resolved.

- It is interesting that the AIFM1-MIA40 complex seems to be strongly required for complex I but rather not for complexes III and IV. Did the authors import the C IV assembly factors COA5, COA6 or CMC2 in AIFM1 mitochondria? As these ones also appear to be dysregulated here (Fig. 1E). Yeast Coa6 e.g. is Mia40-dependent (Vögtle 2012 MPC).

Tissue-specific depletion of AIFM1 also results in tissue specific defects of different respiratory chain complexes with complex I being affected at all times. In HEK293 cells, complexes III and IV were not affected by AIFM1 loss despite the fact that levels of assembly factors/subunits of these complexes were reduced. Our interpretation was that in HEK293 cells, there might be sufficient reserve capacity of these factors to allow assembly of complexes III and IV to proceed normally.

Using isolated mitochondria from HEK293 cells, we now also tested the import of COA6 and CMC2 (see below). Both substrates were as expected slightly affected, clearly less compared with NDUFS5 but more than NDUFB7, NDUFA8 and NDUFB10. In intact cells, these differences might well translate to a reduction of the levels of both proteins. This might become relevant for tissues in which both proteins are limited in their amounts and then also translate into changes in complex III and IV levels. We would like to emphasize that also the dependency on MIA40 differs for different substrates in human cells (see e.g. Habich et al Cell Reports 2019).

Also here, we decided to present this data in the response to the referees section only, since we considered addressing the minor effects on the CIV assembly factors as beyond the scope of the present story and did not want to add further data and expand to include another level of complexity.
Referee #2:

The mitochondrial MIA pathway with MIA40/CHCHD4 mediates the import and oxidative folding of many intermembrane space (IMS) proteins. In the present study, Salscheider et al. report that AIFM1 forms a stable complex with MIA40, which could be important for the import of not only MIA40 itself but also some MIA-pathway substrates including NDUFS5, which facilitates assembly of complex I in the mitochondrial inner membrane. This finding is of high interest because it suggests the existence of previously overlooked aspects, other than the protein level and redox state, of MIA40 that are important for the operation of the MIA-pathway import. The experiments were carefully designed, the employed experimental techniques are very high, and interpretation of the obtained results is appropriate. However, it is unclear how the AIFM1-MIA40 complex, which requires AIFM1 dimerization, affects the import of some MIA pathway substrates, and there was no attempt to address this question experimentally. Localization of MIA40 to the inner membrane by AIFM1 could decrease the efficiency of MIA40 receiving its substrates from the TOM complex, but this is contrary to the results shown here.

Since this work is rich in the data on the roles of NDUFSS in the complex I assembly, the manuscript could be altered to the one fitting better for the EMBO J; the title and direction could be changed to those addressing the above line of questions rather than the still-elusive new role of the AIFM1-MIA40 complex in its import of some substrate proteins.

We thank this referee for the positive assessment of our work and for the constructive comments.

While we agree that our original title was a bit broad, we feel that the manuscript itself was already very much focussed on the complex I assembly defect in AIFM1 knockout cells. As suggested by the referee we changed to the title “A permanent AIFM1-MIA40/CHCHD4 complex drives complex I assembly through efficient import of NDUFSS”, emphasizing more the specific effect of MIA40 and AIFM1 on complex I and NDUFSS biogenesis.

Specific points:

- Page 3, lines 5-6: AIFM1-mediated import of MIA40/CHCHD4 relies on the N-terminal region in MIA40/CHCHD4 and the presence of the dimeric form of AIFM1 (Hangen et al., 2015). It looks that the dimerization of AIFM1 is not described in Hangen et al., 2015.

We thank the referee for this comment. It is correct that in the paper by Hangen et al, the specific interaction site in AIFM1 was not identified, and it was not demonstrated that dimerization of AIFM1 was required for MIA40 binding. Thus, to be precise, we removed the statement on the reliance of MIA40 import on dimeric AIFM1 from the introduction. We now highlighted the fact that MIA40 interacts with an AIFM1 dimer in the discussion.

Notably, Hangen et al demonstrated that AIFM1-MIA40 binding was enhanced by NAD(P)H addition which drives AIFM1 dimerization. Moreover, they showed that interaction was disturbed between MIA40 and AIFM1 G308E, a variant with problems in NAD(P)H binding and thus dimerization. Based on this and our gel filtration data (with cell lysates and in vitro), we think it is justified to assume that only dimeric AIFM1 Interacts with MIA40.
- Fig. S1A: It is difficult to see the shapes of mitochondria due to poor resolution.

We repeated the experiment and included a new version in the revised manuscript (Figure S1A). The colocalization of MIA40 with mitochondria is visible in all cell lines. Enclosed please find an enlarged version of the figure.

- Fig. S1B: The figure showed that the shift of MIA40 to the oxidized state was delayed in AIFM1 KO cells, but in the main text, it is described as "almost unchanged".

This is correct – the kinetics of import as measured with the sensitive pulse chase assay revealed that MIA40 oxidation was slightly delayed; however without affecting MIA40 steady state levels. We now state in the text: “MIA40/CHCHD4 maturation kinetics were slightly delayed which did however not translate into steady state changes in MIA40/CHCHD4 levels (Appendix Figure S1B).”

- Fig. S1C: Why do the total intensities of the bands in each lane differ so much?

We observe this often in maleimide shift assays. For the interpretation of the assay, the ratio between bands in the same lane is important. In Figure S1C, lane 3 represents the steady state redox state of MIA40 in the different cell lines. The ratio of the bands representing the oxidized and reduced CPC motif of MIA40 is very similar in all cases.
- Page 8, lines 27-28: "Notably, NDUFS5 was also absent from the dimeric PP- b/PD-a/TMEM70/TMEM126A/TMEM186/COA1 complex at ~1250 kDa." Which figure does this description refer to?

*This statement refers to Appendix Figure S6F. We added this reference to the sentence.*

- Fig. 3: Pulse-chase experiments for NDUFB7 and NDUFS5. As NDUFB7 is described as "structurally most similar to NDUFS5" (Page 9, line 15), diagrams showing the domains and Cys residues of NDUF7 and NDUFS5 are required.

*Both proteins are the most similar proteins among the four MIA40 substrates in complex I with respect to their disulphide-containing helix-loop-helix structure. We now added a diagram showing the structures to Figure 3A.*

- Fig. 4D: Apparent MWs of the complexes should be shown.

*We added the apparent MWs of the complexes to Figure 4D.*

- Fig. 5E: Why does the ratio of AIFM1 in the complex with MIA40 and in the free form differ among different tissues? Do the expression levels of NDUFS5 and levels of complex I differ among different tissues?

*Indeed, the levels of AIFM1, MIA40 and NDUFS5 differ between different tissues (see e.g. Jian et al, Cell 2020). Importantly, essentially all MIA40 is present in the complex with AIFM1, while AIFM1 appears to be present at superstoichiometric levels in different tissues where it is then also present in the “free form”. At present, we do not know whether this “free” AIFM1 fulfils different functions. It is one line of our future research to explore whether different levels of AIFM1 and MIA40 result in tissue-dependent differences in function of the AIFM1-MIA40 complex or whether AIFM1 fulfils additional functions in tissues where it is present in superstoichiometric excess over MIA40.*
Thank you for submitting your revised manuscript. We have now received comments from the referees (copied below), and I am happy to say that they now support publication. Therefore I would ask you to please resolve a number of editorial issues that are listed in detail below. Please use the document that the data editors have added their comments to for any changes (see below).

Please feel free to contact me if you have further questions regarding the revision or any of the specific points. Once these final issues are resolved, we will be happy to formally accept the work for publication in The EMBO Journal.

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Referee #1:

The authors clarified all points that I have raised. The manuscript is in my opinion now ready for publication.

Referee #2:

This is a revised version of the manuscript previously submitted to EMBO J. Now the authors addressed my concerns and the manuscript was altered appropriately. Therefore the manuscript is in good shape for publication.
Point-by-point response to editorial comments:

1) Please provide a synopsis (image and) text:
   a) Three to four 'bullet points' highlighting the main findings of your study
   b) A short 'blurb' text summarizing in two sentences the study (max. 250 characters). Bullet points and standfirst text should be submitted as a separate manuscript file in LaTeX, RTF or MS Word format.
   (c) image is uploaded)

We provide now synopsis picture, bullet points and blurb

2) Please reduce the number of keywords to a maximum of five, which may or may not appear in the title, should be given in alphabetical order, below the abstract, each separated by a slash (/)

Number of keywords has been reduced to 5, and sorted alphabetically

3) Please update the Conflict of Interest statement to "Disclosure and Competing Interests Statement" and explicitly state if there is nothing to declare.

Done.

4) Please update the reference format to 10 authors et al. in alphabetical order.

Done.

5) Please add reference to Figures Appendix S1A, S1C and S4A. All figures and their individual panels must be referred to in the main text.

Done.

6) Please update the appendix pdf file to the final version (this file will not be typeset and be published as you submit it):
   - Please update the nomenclature of Figures and Tables to Appendix Figure S1, Appendix Figure S2,...
and Appendix Table S1, etc. in the table of contents and the respective legends.

Done.

7) Please ensure that the funding listed in the manuscript is identical to that entered in the submission system. Currently funding is missing in the system.

Done.

8) Please upload the proteomics data as Dataset EV1 and add a legend, for example as a separate tab in the workbook. Please remember to update all references to this in the main manuscript.

Done.

9) The section "In organello import assay" is highly similar to the respective section in Murschall et al. BMC Biology (2020) 18:96. Please cite accordingly or revise the text.

Indeed, this section is very similar as the mentioned article is by my group and we performed exactly the same import experiments for this manuscript. We now cite the indicated manuscript Murschall et al. BMC 2020. We still present the extended explanation of the method.

10) Our data editors have raised several queries with the data descriptors in the figure legends, which you will find as comments in the Word document EMBOJ-2022-110784_figure_QC.docx (as part of your submission and attached to this message). I would appreciate if you incorporated the requested final text modifications and answered the figure legend queries directly in this version, uploading the edited main text document upon resubmission with changes/additions still highlighted via the "Track changes" option.

The comments in the data edited file have all been answered.
Thank you for submitting the final revised version of your manuscript and addressing the remaining points. You will likely hear from me again soon regarding final textual edits of the transfer files, but for now I am happy to inform you that we have formally accepted your study for publication in The EMBO Journal.
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 authorship 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 (e.g. cell line, species name).
     - The assay(s) and method(s) used to carry out the reported observations and measurements in an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
     - 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 2-t tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
       - A statement of how 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.
     - Definitions of statistical methods and measures:
       - An explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
       - The assay(s) and method(s) used to carry out the reported observations and measurements in 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.

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 (not 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 performed experiments in at least three biological replicates, the n numbers are indicated in the figure legends for each experiment. We did not use any statistical test to predetermine the sample size. The sample size was chosen on the basis of our experience and good laboratory practice. |
| --- | --- |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | NA |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | No data 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. | NA |
| 4. For animal studies, include a statement about randomization even if no randomisation was used. | NA |
| 4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinded to the investigators)? If yes please describe. | All data were included; no groups/sets were preferred |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | NA |
| 5. For every figure, are statistical tests justified as appropriate? | NA |
| 6. Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | NA |
| 7. Is there an estimate of variation within each group of data? | Yes, we present data with the Standard Deviation (SD) or present all data points. |
| 8. Is the variance similar between the groups that are being statistically compared? | NA |

C- Reagents
| **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. |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. |
| 10. We recommend consulting the ARRIVE guidelines (see link list at top right) (Pulli Bod. -864) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under ‘Reporting Guidelines’. Please confirm compliance. |

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

| **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 GSE185402, Proteomics data: PRIDE PPD000028 etc.). Please refer to our author guidelines for ‘Data Deposition’. |
| 19. 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. Data sets are fully presented in the study or in a raw data excel file. |
| 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). |
| 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 formats (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 BioModels (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. |

| **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. |

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