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Syncytia formation by SARS-CoV-2 infected cells.

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 7th Sep 2020

Dear Olivier,

Thanks for submitting your manuscript to The EMBO Journal. I am sorry for the delay in getting back to you, but I have now received the two reports on your paper.

As you can see from the comments below, both referees find the analysis interesting and we are interested in considering a revised version of your manuscript. However, referee #2 also raises the issue that the results seen on syncytia formation is done in the context of ACE2 overexpression and that it would be good to have more data supporting the endogenous role of ACE2 in this process. Do you have more data on hand to address this issue? Would be good to discuss this further. Happy to do so via email or a video call.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
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Thank you for the opportunity to consider your work for publication. I look forward to discussing your revisions further with you

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
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- individual production quality figure files (one file per figure)

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 6th Dec 2020.

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Referee #1:

Buchrieser and colleagues have examined SARS-CoV2 induced formation of multinucleated syncitia. They examine the roles of ACE2, TMPRSS2 and IFITM proteins in this activity and show that IFITM proteins suppress fusion, and are thus protective, but TMPRSS2 activity and SPIKE cleavage overcomes IFITM activity to drive viral infection induced syncitia formation.
This is a nicely presented, well controlled and important study. The movies are particularly compelling.

I have only minor suggestions for improvement of clarity

1. The labelling for the figs is a little confusing in places. Can I advise that the authors don't refer to unmodified cells as WT because typically this is used to refer to virus. Better to use "unmodified". Equally the use of control as a label is unclear. Perhaps use "not infected NI" throughout for clarity. I'm referring to the cell panels particularly.

2. I found the description of Fig2C confusing because the authors state that the syncitia do not incorporate IFITM1+ cells, yet there's a bar in 2C for IFITM1 incorporation. The point is that IFITM1 incorporation is reduced, not absent. Please reword for clarity.

3. There are no data presented to support the claims in the second para page 6, ie other cell types. Its cited as data not shown. I think this should be included or not mentioned, particularly given these data are discussed prominently. I agree with the authors that this is an important observation and so, if its compelling why not include, for example in supp data.

4. In 4C IFITM proteins are flag tagged to enable detection by WB. Can we be sure that the Tag

does not impact the sensitivity of IFITM activity to TMPRSS2? Are any of the functional experiments performed with the flag tagged proteins. This is important as the Tag may impact function and/or sensitivity to antagonism. Please check with a functional experiment with Tagged IFITM protein or point out in this section which functional IFITM experiment contains tagged protein.

Referee #2:

The manuscript submitted by Buchrieser, et al. is an interesting study that explores the requirements for syncytia formation using cell culture models and demonstrates a role for both TMPRSS2 and IFITM proteins in modulating cell fusion. The final figure of the manuscript utilizes a clever strategy with donor (spike-expressing) and acceptor (ACE2-expressing) cells and a split GFP expression system. Using this system, the authors show that TMPRSS2 and the IFITM proteins affect fusion when co-expressed in ACE2-expressing acceptor cells but have little effect when co-expressed in spike-expressing donor cells. These experiments shed light on the mechanism of factors affecting syncytia-formation mediated by the SARS-CoV-2 spike protein. Overall, this manuscript is intriguing and certainly some of these experiments required a cell culture system in which different components mediating fusion were expressed in subpopulations of cells; thus, the authors could not use naturally-permissive ACE2-expressing cell lines. However, it is not clear why other experiments were performed almost exclusively in the context of ACE2 overexpression, rather than in cell lines that express endogenous ACE2.

Major Points:

1. Although the authors are using full-length virus to characterize syncytia formation, rather than only SARS-CoV-2 spike expression or pseudotyped virus, the cell lines being used are not naturally permissive and are done in the context of ACE2 overexpression. It seems likely that overexpression of ACE2 may influence the formation of syncytia in a way that does not occur in a more relevant context of infection. The authors do use Vero E6 cells at one point, which are naturally permissive, but do not see formation of syncytia in these cells.

2. Of the cell lines tested that overexpress ACE2, only U2OS and 293 cells form syncytia, but not A549 cells. Given that A549 cells more closely reflect the target host cell in vivo, it brings into question whether the experiments with ACE2 overexpression are problematic.

3. There are several cell lines that are permissive to SARS-CoV-2 infection, including Calu3 and Caco2 cells. Can syncytia formation and the role of IFITMs and TMPRSS2 be evaluated in the context of these cells? Or in the context of primary cell infection?

4. The effects of IFITM and TMPRSS2 expression on syncytia formation are intriguing. However, these experiments are also performed only in the context of overexpression. Using CRISPR/Cas9 mediated knock-outs of IFTIM proteins, or RNA-mediated depletion, would further support a role for these proteins in regulating syncytia formation.

Dear Editor,

We would like to thank you for your consideration of our manuscript entitled “Syncytia formation by SARS-CoV-2 infected cells” for publication in The EMBO Journal. We are delighted by the interest shown by both you and the reviewers. We readily concede that the reviewers’ criticisms improve the clarity and the scientific rigor of our manuscript and we thank them for their diligence. We have responded to reviewers by incorporating new experimental data, rewording for clarity and incorporating a few sentences to further discuss the limitations of our work. Below we address each of the reviewers concerns in point-by-point form:

Referee #1:

Buchrieser and colleagues have examined SARS-CoV2 induced formation of multinucleated syncitia. They examine the roles of ACE2, TMPRSS2 and IFITM proteins in this activity and show that IFITM proteins suppress fusion, and are thus protective, but TMPRSS2 activity and SPIKE cleavage overcomes IFITM activity to drive viral infection induced syncitia formation. This is a nicely presented, well controlled and important study. The movies are particularly compelling.

We sincerely appreciate Referee #1’s positive disposition towards our work and we hope that we are able to sufficiently address all of their concerns through the changes we outline below.

I have only minor suggestions for improvement of clarity

1. The labelling for the figs is a little confusing in places. Can I advise that the authors don't refer to unmodified cells as WT because typically this is used to refer to virus. Better to use "unmodified". Equally the use of control as a label is unclear. Perhaps use "not infected NI" throughout for clarity. I'm referring to the cell panels particularly.

We thank the reviewer for bringing this to our attention and we have clarified the labelling as they have suggested. We now reserve the label of “WT” to refer only to virus and we have also changed “control” to “not infected (NI)” in the cell panels when referring to infection experiments. However, we have a slight reservation on using “unmodified’ to refer to the cell lines, as they often have been modified through the transduction of the GFP-split reporter system. In the closed context of the experiment where reporter cells are transduced with IFITMs and then compared to cells transduced with a control vector, we believe that “control” is the most suitable term. In order to avoid any possible misunderstanding, we have included in brackets beside “control cells”: “transduced with control vector”

2. I found the description of Fig2C confusing because the authors state that the syncitia do not incorporate IFITM1+ cells, yet there's a bar in 2C for IFITM1 incorporation. The point is that IFITM1 incorporation is reduced, not absent. Please reword for clarity.

We have clarified that IFITM1 incorporation into syncytia is not absent but reduced. The description for Figure 2C now reads “The fusion of IFITM1+ cells with U2OS-ACE2 syncytia is drastically reduced”.

3. There are no data presented to support the claims in the second para page 6, ie other cell types. Its cited as data not shown. I think this should be included or not mentioned, particularly given these data are discussed prominently. I agree with the authors that this is an important observation and so, if its compelling why not include, for example in supp data.

We had initially only mentioned the result in passing as we felt these results were tangential to the story. With the reviewer’s prompt, we continued to pursue this line of investigation and were pleasantly surprised with the results. As mentioned in the manuscript Vero cells do not form syncytia upon infection (Fig EV7A) and this was also true for our initial experiments with A549-ACE2 cells. However, upon using a clonal population of A549-ACE2 cells we were able to clearly visualize infection induced syncytia formation (Fig EV6B). The discrepancy is likely due to the progressive loss of ACE2 in the older A549-ACE2 cells over time. We had also continued experiments with Vero cells and found that upon transfection of the spike protein or in the context of an acceptor/donor co-culture system with 293T cells expressing spike, they are capable of forming syncytia (Fig EV7). These important results show that cells with endogenous levels of ACE2 (Vero cells) are able to fuse when they are in contact with S-expressing cells or with infected cells. We have included all of this data in the supplement and have modified the manuscript (Page 6, Paragraph 2) which now reads:

“We next assessed whether other cell types form syncytia upon SARS-CoV-2 infection. To this aim, we used 293T cells transfected with ACE2 and also generated A549-ACE2 cells. The two cell lines readily formed syncytia upon infection (Fig EV6A-B). In order to rule out the possibility that syncytia formation is solely dependent on ACE2 over-expression, we investigated the naturally permissible Vero cells with the GFP-split system. We did not detect fused infected Vero cells (Fig EV7A), thus we used as donors U20S-ACE2 infected cells, that we co-cultivated with uninfected Vero cells. Numerous heterocellular syncytia were formed in a short period of time (8h) (Fig EV7D). The ability of Vero cells to fuse was again confirmed when donor 293T cells were transfected with S and co-cultivated with Vero acceptor cells (Fig EV7C). Additionally, Vero cells are also capable of forming syncytia upon transfection of only the S protein (Fig EV7B). Of note, Caco2 cells did not fuse upon SARS-CoV-2 infection (Fig EV6D). Taken together, our data strongly suggest that the ability to form syncytia upon SARS-CoV-2 infection is dependent on cell type as well as on the surface levels of S and ACE2. Fusion is detected in Vero cells with endogenous levels of ACE2.”

4. In 4C IFITM proteins are flag tagged to enable detection by WB. Can we be sure that the Tag does not impact the sensitivity of IFITM activity to TMPRSS2? Are any of the functional experiments performed with the flag tagged proteins. This is important as the Tag may impact function and/or sensitivity to antagonism. Please check with a functional experiment with Tagged IFITM protein or point out in this section which functional IFITM experiment contains tagged protein.

We understand the reviewer's concern that tags may alter the function of IFITM proteins but we would like to suggest that our previous work, as well as those from various groups with the same type of constructs demonstrate that the tag is quite benign. We have shown that tagged and untagged IFITM proteins behave similarly and prevent the fusion and spread of HIV (Compton et al., 2014; Cell Host & Microbe PMID: 25464829) and are also capable of inhibiting the formation of placental syncytiotrophoblast (Buchrieser et al., 2019; Science PMID: 31296770). However, we share the reviewer's desire to avoid any possible misunderstanding, so we have included the following statement in the materials and methods (Page 9 Paragraph 4: Plasmids):

"The IFITM plasmids used throughout this study, either in transfections or in the generation of stably expressing cell lines, contain a FLAG tag on the N terminus."

Referee #2:

The manuscript submitted by Buchrieser, et al. is an interesting study that explores the requirements for syncytia formation using cell culture models and demonstrates a role for both TMPRSS2 and IFITM proteins in modulating cell fusion. The final figure of the manuscript utilizes a clever strategy with donor (spike-expressing) and acceptor (ACE2-expressing) cells and a split GFP expression system. Using this system, the authors show that TMPRSS2 and the IFITM proteins affect fusion when co-expressed in ACE2-expressing acceptor cells but have little effect when co-expressed in spike-expressing donor cells. These experiments shed light on the mechanism of factors affecting syncytia-formation mediated by the SARS-CoV-2 spike protein. Overall, this manuscript is intriguing and certainly some of these experiments required a cell culture system in which different components mediating fusion were expressed in subpopulations of cells; thus, the authors could not use naturally permissive ACE2-expressing cell lines. However, it is not clear why other experiments were performed almost exclusively in the context of ACE2 overexpression, rather than in cell lines that express endogenous ACE2.

We appreciate the reviewer's interest and attention to our work, and have endeavoured to address their concerns regarding the relationship between ACE2 overexpression and syncytia formation by providing more experimental data on syncytia formation in naturally permissive cell lines (Vero and Caco2) and by clarifying our claims in the text to more precisely describe our observations.

Major Points:

1. Although the authors are using full-length virus to characterize syncytia formation, rather than only SARS-CoV-2 spike expression or pseudotyped virus, the cell lines being used are not naturally permissive and are done in the context of ACE2 overexpression. It seems likely that overexpression of ACE2 may influence the formation of syncytia in a way that does not occur in a more relevant context of infection. The authors do use Vero E6 cells at one point, which are naturally permissive, but do not see formation of syncytia in these cells.

We appreciate the reviewers request that we provide a comprehensive understanding on the relationship between ACE2 overexpression and syncytia formation. We have followed up on this

request by preforming several co-culture experiments with the naturally permissive Vero cells; the results of which show that it is possible to induce fusion in non-ACE2 over expressing cells. However, we did not detect syncytia in Caco2 cells (Fig EV6D), indicating that the ability to of infected cells to form syncytia is cell type dependent.

We have modified the manuscript in order to describe our new findings (Page 6 Paragraph 2):

"We next assessed whether other cell types form syncytia upon SARS-CoV-2 infection. To this aim, we used 293T cells transfected with ACE2 and also generated A549-ACE2 cells. The two cell lines readily formed syncytia upon infection (Fig EV6A-B). In order to rule out the possibility that syncytia formation is solely dependent on ACE2 over-expression, we investigated the naturally permissible Vero cells with the GFP-split system. We did not detect fused infected Vero cells (Fig EV7A), thus we used as donors U20S-ACE2 infected cells, that we co-cultivated with uninfected Vero cells. Numerous heterocellular syncytia were formed in a short period of time (8h) (Fig EV7D). The ability of Vero cells to fuse was again confirmed when donor 293T cells were transfected with S and co-cultivated with Vero acceptor cells (Fig EV7C). Additionally, Vero cells are also capable of forming syncytia upon transfection of only the S protein (Fig EV7B). Of note, Caco2 cells did not fuse upon SARS-CoV-2 infection (Fig EV6D). Taken together, our data strongly suggest that the ability to form syncytia upon SARS-CoV-2 infection is dependent on cell type as well as on the surface levels of S and ACE2. Fusion is detected in Vero cells with endogenous levels of ACE2."

2. Of the cell lines tested that overexpress ACE2, only U2OS and 293 cells form syncytia, but not A549 cells. Given that A549 cells more closely reflect the target host cell in vivo, it brings into question whether the experiments with ACE2 overexpression are problematic.

We thank reviewer #2 for his/her comment. We initially reported that A549-ACE2 cells do not form syncytia, but further investigations have shown that our uncloned cell population had progressively lost ACE2 expression. We thus isolated individual clones of A549-ACE2 cells. The new A549-ACE2 cells readily formed syncytia, as now shown in Fig. SX (Fig EV6B). It is likely that our previous cells suffered from reduction/loss of ACE2 expression as a consequence of drift.

3. There are several cell lines that are permissive to SARS-CoV-2 infection, including Calu3 and Caco2 cells. Can syncytia formation and the role of IFITMs and TMPRSS2 be evaluated in the context of these cells? Or in the context of primary cell infection?

In order to accommodate the reviewer's request, we expanded our investigation to include Vero, Calu3 and Caco2 cell lines. Vero cells, with endogenous levels of ACE2, formed syncytia (Fig EV7). Calu3 cells were permissible to viral infection, but they proved to be a difficult model for studying syncytia formation, as they naturally exhibit a syncytia-like morphology, even without infection. We have thus not included these inconclusive results in the manuscript. Caco2 cells were also sensitive to infection, but we did not detect syncytia in infected cells (Fig EV6D). We did not further explore whether Caco2 express insufficient surface levels of S, ACE2 or other molecules, since this would have been redundant to our new experiments with Vero cells.

We understand that the reviewer is concerned about the relevance of our work. We respectfully submit that our study provides a cellular perspective on the observed phenomenon of pneumocytes forming syncytia in the lungs of severely infected patients (Giacca et al., 2020 MedRxiv https://doi.org/10.1101/2020.06.22.20136358). We agree that primary lung cells would be an effective investigative tool for the future.

4. The effects of IFITM and TMPRSS2 expression on syncytia formation are intriguing. However, these experiments are also performed only in the context of overexpression. Using CRISPR/Cas9 mediated knock-outs of IFTIM proteins, or RNA-mediated depletion, would further support a role for these proteins in regulating syncytia formation.

We understand the reviewer's desire to have our model reflect a more in vivo situation and we acknowledge that the lack of endogenous IFITM systems is a limitation of our study. Other recent investigations have provided contrasting evidence regarding the role of IFITMs n SARS-CoV-2 infection, with two unreviewed reports posted in bioarXiv showing that IFITMs may either restrict or promote infection (Shi et al., 2020 https://doi.org/10.1101/2020.08.11.246678) (Bozzo et al., 2020 https://doi.org/10.1101/2020.08.18.255935). We have so far focused our work on the formation of syncytia by infected cells, whereas the two other manuscripts rather study the impact of IFITMs on infection with cell-free viral particles. We hope to further elucidate the relationship between endogenous IFITMs and syncytia formation in our future work. We also previously reported that IFITM levels in our cell systems are similar to those observed in cells treated with type-I IFN (Compton et al., 2014; Cell Host & Microbe PMID: 25464829) (Buchrieser et al., 2019; Science PMID: 31296770).

We have added the following statement to our discussion in order to address this limitation of our study:

"Furthermore, recent reports have suggested that IFITMs may either enhance or restrict SARS-CoV-2 infection, depending on the experimental system and the cell type (Bozzo, Nchioua et al., 2020, Shi, Kenney et al., 2020). Future investigations into the role endogenous IFITMs and TMPRSS2 conducted with primary cells treated or not with type-I IFN, will provide a more thorough translational understanding of viral induced syncytia formation."

1st Revision - Editorial Decision 6th Oct 2020

Dear Olivier,

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by referee #1. As you can see from the comments below, the referee appreciates the introduced changes and supports publication here. I am therefore very pleased to accept the manuscript for publication here.

Before sending you the formal acceptance letter there are just a few minor things we have to resolve.

- The movie legends should be removed from the main MS file. Each movie should be zipped with its legend and uploaded as a separate file

- We require a data availability section. As far as I can see no data needs to be deposited in an external database. If correct please then state This study includes no data deposited in external repositories.

- Are the same Hoechst images u=used in Fig 2 EVA&C panels? If so the please mention this in the figure legends

- For the funding info - I just want to double check with you if you need to add funding numbers of the INSERM, REACTing and EU (RECOVER) grants. Please take a look.

- We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data edited manuscript file". Please take a look at the word file and the comments regarding the figure legends and respond to the issues.

That should be all - you can use the link below to submit the revised version.

Congratulations on a great study!

Best Karin

Karin Dumstrei, PhD
Senior Editor
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Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

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- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 4th Jan 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The authors have addressed my concerns with new data which are compelling or new text to answer to my questions. It is my view that this manuscript is complete and ready for publication.

- The movie legends should be removed from the main MS file. Each movie should be zipped with its legend and uploaded as a separate file

The legends have been removed from the manuscript and zipped as separate files with the movies.

- We require a data availability section. As far as I can see no data needs to be deposited in an external database. If correct please then state This study includes no data deposited in external repositories.

A data availability section has been added and states: "This study includes no data deposited in external repositories."

- Are the same Hoechst images used in Fig 2 EVA&C panels? If so the please mention this in the figure legends

Yes, the same Hoechst images are used in figure EV2 A and C. A sentence has been added to the figure legends stating the same images were used in EV2 A and C.

- For the funding info - I just want to double check with you if you need to add funding numbers of the INSERM, REACTing and EU (RECOVER) grants. Please take a look.

No funding numbers are required.

- We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

A pdf of the uncropped western blot gels was added as supplementary "source data".

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data edited manuscript file". Please take a look at the word file and the comments regarding the figure legends and respond to the issues.

We checked the file and responded to the comments. The new uploaded manuscript contains the track changed edits and responses.

2nd Revision - Editorial Decision 8th Oct 2020

Dear Olivier,

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

Congratulations on an exciting study!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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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)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Three to nine independent replicates were done for each experiment to ensure sufficient statistical power
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?	NA
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.	Images were processed using automated analysis on all conditions simultaniously to avoid subjective bias.
For animal studies, include a statement about randomization even if no randomization 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. blinding of the investigator)? If yes please describe.	No, but imaging analysis were performed automatically to avoidinvestigator bias.
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?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Flow cytometry analyses are performed on upt to 10,000 cells with a normal distribution, as visualized on the histograms

Is there an estimate of variation within each group of data?	Each point is the mean of triplicates. The SD allows an estiamtion of the variation. Each experiment is representative of at least three independent ones. The mean and SD of the three experiments is then calculated
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).	All antibody catalog number and/or clone number are indicated in the Material and methods. Negative and positive controles for each of the antibodies is provided within the figures and supplementary data.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines were tested for mycoplasma on a bi-weekly basis and found negative. Origin of cells is indicated in the manuscipt material and methods.

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

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).	Representative experiments are shown in the main or supplementary figures. Raw data are availabke upon request
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