THE EMBO JOURNAL

Unbiased proteomic profiling of host cell extracellular vesicle composition and dynamics upon HIV-1 infection

Clotilde Thery, Lorena Martin-Jaular, Nathalie Névo, Julia Schessner, Mercedes Tkach, Mabel Jouve, Florent Dingli, Damarys Loew, Kennethw Witwer, Matias Ostrowski, and Georg Borner
DOI: 10.15252/embj.2020105492

Corresponding author(s): Clotilde Thery (clotilde.thery@curie.fr) , Georg Borner (borner@biochem.mpg.de)

Review Timeline:

Submission Date:	2nd May 20
Editorial Decision:	2nd Jul 20
Revision Received:	2nd Nov 20
Editorial Decision:	21st Dec 20
Revision Received:	25th Jan 21
Accepted:	26th Jan 21

Editor: Daniel Klimmeck

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.)

1st Editorial Decision 2nd Jul 2020

Dear Clotilde,

Thank you again for the submission of your manuscript (EMBOJ-2020-105492) to The EMBO Journal and in addition providing us with a preliminary revision plan. Thank you also for your patience with my response, which got delayed due to detailed discussions in the team regarding your point-by-point response. As mentioned earlier, your study has been sent to three referees, and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and novelty of your approach and findings, although they also express major concerns. Referees #2 and #3 raise reservations regarding the biological advance provided and in vivo relevance of your dataset. Further, reviewer #3 points to methodological issues related to the EV analytical protocols applied and overlap of markers between different fractions. Referee #1 requests additional controls to corroborate unique markers highlighted and in addition states that both utility of the pattern analysis presented for conclusions on specific EV fractions and completeness as methods standard need consolidation.

Given the interest stated and broader angle of your approach and findings, we are happy to invite you to revise your manuscript experimentally to address the referees' comments, along the lines sketched in your outline.

Please note that as discussed, we consider your work as a resource-type article, and thus while well taken, the reviewers' concerns on biological or mechanistic advance are not at the core of our consideration for this study.

Further, we agree that consolidating the neighborhood pattern analysis results with additional biochemical data on specific markers will be an important aspect.

We did consider your argument made regarding feasibility of the primary T cell EV extraction. We encourage you to execute these experiments, as it would be important to make a case for the in vivo pathophysiological relevance of your dataset.

Finally, we recently introduced Structured Methods a new format for the Materials and Methods of articles published at EMBO Press. Adhering to this format is optional for research articles. However, considering the strong methodological aspect of your study, we would strongly encourage you to use it. Specifically, the Material and Methods section should include a Reagents and Tools Table (listing key reagents, experimental models, software and relevant equipment and including their sources and relevant identifiers) followed by a Methods and Protocols section in which we encourage the authors to describe their methods using a step-by-step protocol format with bullet points. More information on how to adhere to this format as well as downloadable templates (.doc or .xls) for the Reagents and Tools Table can be found in the author guidelines of our sister journal Molecular Systems Biology http://msb.embopress.org/authorguide#materialsandmethods.
An example of a paper with Structured Methods can be found here: http://msb.embopress.org/content/14/7/e8071. We encourage you to be even more explicit in adding details on the experimental procedures, as this should be valuable in ensuring reproducible application of the approach in other cellular systems.

Please feel free to contact me if you have any questions or need further input on the referee comments.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Kind regards,

Daniel

Daniel Klimmeck, PhD
Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

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:
http://embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see http://msb.embopress.org/authorguide#dataavailability).

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Method) that follows the model below (see also http://emboj.embopress.org/authorguide#dataavailability). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

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).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (http://emboj.embopress.org/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
http://emboj.embopress.org/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

Referee #1:

In this manuscript, the authors have designed a proteomics-based methodology to examine the protein composition of extracellular vesicle (EV) subtypes. This is achieved by combining classical ultra-centrifugation with SILAC labeling and mass spectrometry, aided by a computational tool to analyze and visualize proteins with shared fractionation profiles. The approach is applied to a HIV infection model to identify infection-induced changes in EV composition. This was then validated for a handful of top-scoring proteins.

The authors pick up a challenging question, aiming to differentiate between EVs and quantify the changes in their protein upon HIV-infection. The problem resides in the fact that purification of EVs devoid of contaminating proteins is very difficult, that separation of EV populations is nearly impossible due to their similarity in size and density, and that density gradients allow EV separation only at low resolution. As a result, the field is fraught with long lists of proteins that are claimed to represent the composition of specific EV classes. The present study takes a different approach, not aiming to determine the total protein composition of EVs, but instead to measure differences in their composition upon a perturbation (here HIV infection). Their method follows a rationale that was previously used to allocate proteins to cellular organelles, namely to group proteins by their similarity in behavior when separating EVs through a series of centrifugation steps: proteins that follow the same trajectory across centrifugation fractions are like to co-exist in the same EV population. This is technically achieved by the quantitative comparison of a given protein relative to a SILAC-labeled protein in a reference sample. The collective results are captured in a relational database, by assigning a similarity score to every protein relative to any other protein that was identified. This may be a useful approximation of co-localization in EVs, however it is prone to both under- and over-interpretation, hence this should be taken with caution (see comments below). Yet, if the authors can provide a proper explanation to future users to avoid mis-interpretation of the visualized data, this approach is more robust, conservative, and quantitative than any method in the field, with the potential to set a new standard. Overall the authors are (rightly) very cautious in their statements throughout the manuscript, e.g. saying that proteins are 'likely' to co-exist in a EVs if they share the same fractionation pattern. On the one hand this should be commended since authors do not over-interpret their data, but on the other hand it demonstrates how hard it is to really prove co-localization, with the result that this has been formally shown in the paper only for a single protein (using co-IP to show co-localization of SPN, MOV10 and p24, while being separate from SERINC3). This makes the biological merit a little thin, however the shown findings for SERINC3 and its causal connection to HIV infection may be valuable for the (HIV) field.

Specific comments:
1. One of my main concerns is that the authors should be more explicit in how the (dis)similarities between fractionation profiles may be interpreted. In particular, perfect overlap of 2 proteins across fractions does not prove that they are in the same EV, since different EVs can have the same size and density. Conversely, if 2 proteins have different profiles, this neither disproves that they are in the same EV, nor does it prove that they are in different EVs, since EV sub-populations may preferentially contain one or the other protein. All this becomes even more complicated when considering that proteins can occur in more than one EV, meaning that the shown protein profiles are the average across many EV types. The authors acknowledge all of this, but this only occurs in the discussion, after having drawn their conclusions in the preceding sections. This means that their conclusions may the most likely interpretation of their observations, however none of them are a formal proof. Clarifying this up-front will better guide the reader in placing the observations and conclusions in the proper context. As mentioned above, this is carefully phrased by the authors, however it remains a weakness of the paper.
2. Related to the comment above, the authors do not have proof that EVs contain either CD63 or CD81 (claimed in the abstract, probably concluded from Fig 2A), since it cannot be excluded that an

overlapping EV population contains both proteins.
3. The authors may consider emphasizing up-front that their approach deviates from the idea aiming to generate a complete list of protein cargo for EV subtypes, as has been attempted by many studies in the past with only limited success. Instead, by designing their experimental set-up to determine changes in EV composition is a novel perspective that helps pointing to biologically relevant events, as shown by the changes induced by HIV infection.
4. When describing their 'neighbourhood network predictor' (p7), authors need to explain how 'neighbourhood' should be interpreted in biological terms. It is understood that proteins with a similar fractionation profile will have a closer neighbourhood than those with different profiles, however a confounding factor in this particular case is that co-fractionation does not necessarily equate to co-occurrence in the same vesicle (see point 1 above). Conversely, poor co-fractionation (and hence poor neighbourhood) does not dis-prove that both proteins cannot co-exist in part of the EV-pool where each of the proteins resides. The risk is that this kind of visualisation, necessarily detached from the underlying raw data, may be easily mis-interpreted to demonstrate physical co-localization as the 'biological truth'. Yet, more interpretations are possible.
5. Fig 1 A and C: In EVs from HIV-infected cells, CD45 is nearly absent in the 10k fraction. How can this be explained?
6. Page 8: When comparing EV composition of control and HIV-infected cells, why were experiments run in parallel? It should have been much more powerful to differentially SILAC-label infected and control cell, mix EVs from both conditions, and perform centrifugation of the combined sample. Importantly, this would allow a direct proteomic comparison of EV fractions within a single experiment, avoiding quantification via an external reference.
7. Fig 3B: why are some of the profiles so difference in the left and right panels? I believe these are supposed to indicate replicates?
8. Fig S3b and page 9: A change in fractionation pattern of a given protein does not necessarily mean that it associates with different EV subtypes, it may also mean that the size/shape/density of the EV itself is altered.
9. Authors describe that MOV10 and SPN move to the virion in infected cells, however a biological role is not discussed or investigated.

Referee #2:

The manuscript describes the protein composition of EVs from control and HIV infected cells. The article contains proteomic data that is better suited for specialized journals. The study lacks biological insights and is preliminary for EMBO J. The study also lacks any in vivo evidence of the claim "re-routed to non-viral EVs". Though the observations may be useful, the results are preliminary and lack biological/functional data.

Referee #3:

In their manuscripts, "Proteomic profiling allows unbiased analysis of HIV-1 and house extracellular vesicles" Martin-Jaular and colleagues employed the SILAC quantitative proteomic profiling approach to analyze the composition of different extracellular vesicle (EV) fractions from Jurkat cell cultures. Using this technique they also analyzed EV compositions after infection with HIV. Their goal was to identify networks of closely interacting proteins (through the neighborhood network predictor) that would distinguish different types of EV and potentially reveal functional differences

that could give insights into the role of pEV in general and HIV-induced EV specifically. The SILAC technology is well established and has been previously used to analyze compositions of vesicular structures of the cell, as for example Clathrin-coated vesicles.

While the technical approach of the manuscript is sound, the results in this lengthy and not easy to follow manuscript are rather disappointing and do not provide much of a new or meaningful insight. The authors state in their discussion: "to date, the majority of proteomic studies have provided a crude list of proteins found in both preparations of EV containing various mixtures of subtypes". While the authors indeed provide more than a crude list, their bioinformatic plots do not give a decisively better insight. Potetially, their vesicle fractionation based on different centrifugation steps (F1-F3) is not sophisticated enough to match the SILAC approach In other words, this approach still creates of lot of overlap between the different types of vesicles and secretion pathways, particularly in the F3 pellet. . On the other hand the SILAC approach may not be sensitive enough (see below). Although we learn that the cellular proteins MOV10 and SPN are found in virus particles and that SERINC3 is shuttled into EV in the presence of Nef, the meaning of these findings remains unresolved. It is at least questionable that HIV uses a whole accessory gene to direct SERIN3 into an EV pathway in order to get rid of a restriction factor. Most likely there is a much bigger picture, at least with respect to Nef-induced EV secretion.

A main problem is a discrepancy with previous findings. Many proteins, particularly signaling proteins, Src kinases and proteases that have previously been identified in EV from HIV patients, HIV primary cell culture models and other in vitro systems were not identified in this analysis. This raises suspicion that either the SILAC approach was not sensitive enough, or the cell culture model with Jurkat cells was not sufficiently representing the in vivo situation. In view of this discrepancy, and in order to validate their approach, the authors should have at least analyzed vesicles from primary T cell cultures and compare these results with the content in plasma extracellular vesicles from HIV patients.

Analyzing only the content of vesicles preparations may not provide the answers the field is hoping to find. It probably takes detailed molecular biology in conjunction with imaging, experimental marker proteins, sophisticated purification techniques and electron microscopy to identify different pathways of vesicle secretion including respective factor contents. For example there is evidence, that vesicle secretion in HIV infection is mediated by the so-called nonconventional secretion pathway (Zhao et al., 2019 Traffic), induced by HIV and mediated by a src kinase. None of the results presented here would point in such a direction. Therefore one has to question whether the here described approach and tool is sufficient to address these complex but state of the art questions.

Point-by-point reply to reviewers' comments

Referee #1:

In this manuscript, the authors have designed a proteomics-based methodology to examine the protein composition of extracellular vesicle (EV) subtypes. This is achieved by combining classical ultra-centrifugation with SILAC labeling and mass spectrometry, aided by a computational tool to analyze and visualize proteins with shared fractionation profiles. The approach is applied to a HIV infection model to identify infection-induced changes in EV composition. This was then validated for a handful of top-scoring proteins.

The authors pick up a challenging question, aiming to differentiate between EVs and quantify the changes in their protein upon HIV-infection. The problem resides in the fact that purification of EVs devoid of contaminating proteins is very difficult, that separation of EV populations is nearly impossible due to their similarity in size and density, and that density gradients allow EV separation only at low resolution. As a result, the field is fraught with long lists of proteins that are claimed to represent the composition of specific EV classes. The present study takes a different approach, not aiming to determine the total protein composition of EVs, but instead to measure differences in their composition upon a perturbation (here HIV infection). Their method follows a rationale that was previously used to allocate proteins to cellular organelles, namely to group proteins by their similarity in behavior when separating EVs through a series of centrifugation steps: proteins that follow the same trajectory across centrifugation fractions are like to co-exist in the same EV population. This is technically achieved by the quantitative comparison of a given protein relative to a SILAC-labeled protein in a reference sample. The collective results are captured in a relational database, by assigning a similarity score to every protein relative to any other protein that was identified.
We appreciate this reviewer's very accurate understanding of the issues we tried to address and the approaches we took.

This may be a useful approximation of co-localization in EVs, however it is prone to both under- and over-interpretation, hence this should be taken with caution (see comments below). Yet, if the authors can provide a proper explanation to future users to avoid mis-interpretation of the visualized data, this approach is more robust, conservative, and quantitative than any method in the field, with the potential to set a new standard.
Thank you for this positive appreciation of our work. See our response to specific point 1 below: we have included a new paragraph in the results section at the end of figure 2, to describe better what minimal interpretations can be taken from the NNP results.

Overall the authors are (rightly) very cautious in their statements throughout the manuscript, e.g. saying that proteins are 'likely' to co-exist in a EVs if they share the same fractionation pattern. On the one hand this should be commended since authors do not over-interpret their data, but on the other hand it demonstrates how hard it is to really prove co-localization, with the result that this has been formally shown in the paper only for a single protein (using co-IP to show co-localization of SPN, MOV10 and p24, while being separate from SERINC3).
This is true indeed. See our response to specific point 2 below: we have performed additional biochemical experiments to isolate and analyze EVs containing CD63 or CD81 or CD3, to validate the compositional analysis for three additional proteins. These results are provided in new figure 3 and figure EV2.

This makes the biological merit a little thin, however the shown findings for SERINC3 and its causal connection to HIV infection may be valuable for the (HIV) field.
We hope that the additional biochemical data, including those done on EVs from primary CD4 T cells (new figure EV2) have increased the biological merit, and that this reviewer will now consider this work worth publishing as a resource article.

Specific comments:
1. One of my main concerns is that the authors should be more explicit in how the (dis)similarities between fractionation profiles may be interpreted. In particular, perfect overlap of 2 proteins across fractions does not prove that they are in the same EV, since different EVs can have the same size and density. Conversely, if 2 proteins have different profiles, this neither disproves that they are in the same EV, nor does it prove that they are in different EVs, since EV sub-populations may preferentially contain one or the other protein. All this becomes even more complicated when considering that proteins can occur in more than one EV, meaning that the shown protein profiles are the average across many EV types. The authors acknowledge all of this, but this only occurs in the discussion, after having drawn their conclusions in the preceding sections. This means that their conclusions may the most likely interpretation of their observations, however none of them are a formal proof. Clarifying this up-front will better guide the reader in placing the observations and conclusions in the proper context. As mentioned above, this is carefully phrased by the authors, however it remains a weakness of the paper.
We thank this reviewer for acknowledging our care not to overinterpret our data. We have included a new paragraph at the end of the results section describing figure 2, EV1 and the NNP (p.8), to describe the minimal conclusions that can be taken from the NNP, and acknowledge that these are not formal demonstrations:
"*The NNP's output does not provide EV proteomes; […] the separate clusters for CD63 and CD81 indicate that there are at least two out of three EV subtypes […] but the NNP alone cannot predict which ones exist."*
We have also extensively re-read our text and made sure to describe the neighbourhood results as suggesting presence in EVs with similar properties, rather than in identical EVs: e.g. replacing "same type of EVs" by "EVs with similar biophysical (pelleting) properties" p.6 and p.7 last paragraphs.
Finally, a new sentence at the end of introduction also clarifies the novelty of the approach and the ways to use it:
"Our approach differs conceptually from previous studies, […] Cluster analysis then indicates groups of proteins likely to be associated with the same EV subtypes, which can be further analysed by orthogonal biochemical methods. […]"

2. Related to the comment above, the authors do not have proof that EVs contain either CD63

or CD81 (claimed in the abstract, probably concluded from Fig 2A), since it cannot be excluded that an overlapping EV population contains both proteins.

This is a very valid comment. We provide now an entirely new set of data, aimed at determining by complementary biochemical approaches the association of CD63, CD81, and CD3 to subtypes of EVs. These results are shown in new figure 3 (Jurkat EVs) and new figure EV2 (same biochemical approaches on primary CD4+ T cells EVs) and described in p.8-10 of the results section.

We performed IP of EVs with either anti-CD63 or anti-CD81, to compare by Western blot the pull-down and flow-throughs in terms of presence of a few proteins identified as specific neighbours of one or the other by our NNP tool. For technical reasons (batch of antibody not working), we could not test an excellent candidate protein of the CD63 network, PDCD6IP = Alix, but we could test syntenin = SDCBP. This analysis shows that CD63+ EVs are in fact a subpopulation of CD81+ EVs, rather than a separate population, and confirms the preferential association of ADAM10 with CD81 and of syntenin with CD63. We have thus changed the abstract sentence to reflect this new result.

We also performed another immuno-capture-based approach called MacsPlexExo, and confirmed the major presence of CD81+ EVs released by Jurkat and primary CD4 T cells, a lower abundance of CD63+ and CD3+ EVs, and prominent presence of ITGB1 = CD29 on the CD81+ EVs. This approach also showed a higher enrichment of a few markers in CD3+ as compared to CD63+ EVs, such as CD45=PTPRC which appeared closer to the network of CD3 rather than the network of CD63 when searching the Neighbour Network Predictor.

We thus conclude that the new data are consistent with the Neighbour Network Predictor (NNP) predictions, and further refine their interpretation.

3. The authors may consider emphasizing up-front that their approach deviates from the idea aiming to generate a complete list of protein cargo for EV subtypes, as has been attempted by many studies in the past with only limited success. Instead, by designing their experimental set-up to determine changes in EV composition is a novel perspective that helps pointing to biologically relevant events, as shown by the changes induced by HIV infection.

Thanks for this suggestion. We have now emphasized better the novelty of our approach, in the final paragraph of the introduction p4, based on this reviewers comment:

"Our approach differs conceptually from previous studies, […] Cluster analysis then indicates groups of proteins likely to be associated with the same EV subtypes, which can be further analysed by orthogonal biochemical methods. As a proof of concept […]"

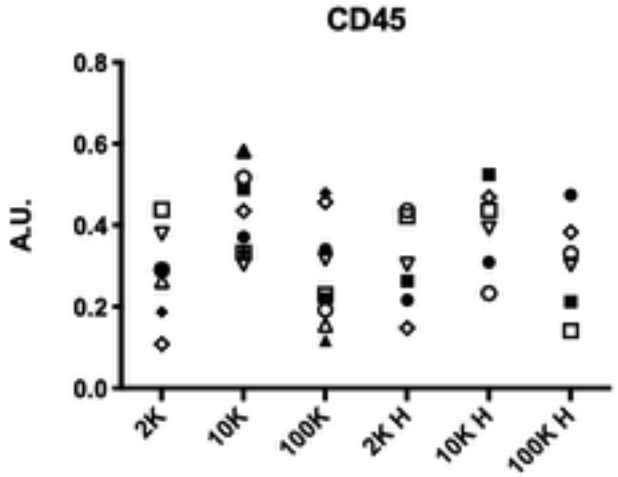
4. When describing their 'neighbourhood network predictor' (p7), authors need to explain how 'neighbourhood' should be interpreted in biological terms. It is understood that proteins with a similar fractionation profile will have a closer neighbourhood than those with different profiles, however a confounding factor in this particular case is that co-fractionation does not necessarily equate to co-occurrence in the same vesicle (see point 1 above). Conversely, poor co-fractionation (and hence poor neighbourhood) does not dis-prove that both proteins cannot co-exist in part of the EV-pool where each of the proteins resides. The risk is that this kind of visualisation, necessarily detached from the underlying raw data, may be easily mis-interpreted to demonstrate physical co-localization as the 'biological truth'. Yet, more interpretations are possible.

We agree with these comments. We have thus included a paragraph at the end of the results section on figure 2, EV1 and the NNP (p.8), to describe the minimal conclusions that can be taken from the NNP, and acknowledge that these are not formal demonstrations:

"*The NNP's output does not provide EV proteomes; […] the separate clusters for CD63 and CD81 indicate that there are at least two out of three EV subtypes […] but the NNP alone cannot predict which ones exist."*

and at the end of the new results on fig3 and fig EV2, another sentence to conclude that ***"****the biochemical analysis validates several predictions of the NNP analysis and further refines their interpretation."*

5. Fig 1 A and C: In EVs from HIV-infected cells, CD45 is nearly absent in the 10k fraction. How can this be explained?
Recovery of material in the 10K fraction is somehow more variable than in the 100K fraction, especially in the HIV-infected situation, probably due to variable induced level of cell death. However, we consistently observed higher amount of CD45 in the 2K and/or 10K than the 100K, as shown below, in this figure for the reviewer: quantification of the CD45 signal in 9 independent experiments, 2K-10K-100K pellets of control, or HIV-infected (H) cells.



We have replaced the image of the Western blots of figure 1A and 1C, to show a more representative image, with CD45 clearly visible in the 10K pellet.

6. Page 8: When comparing EV composition of control and HIV-infected cells, why were experiments run in parallel? It should have been much more powerful to differentially SILAC-label infected and control cell, mix EVs from both conditions, and perform centrifugation of the combined sample. Importantly, this would allow a direct proteomic comparison of EV fractions within a single experiment, avoiding quantification via an external reference.
This is an interesting idea, and we (G Borner) have tried this in the past for the intracellular vesicles. Unfortunately, it does not work at all, for technical reasons: first, without the internal standard, there are too many missing data points; second, it reduces the analysis to finding differences. Since most proteins will not change distribution, they will have profiles with ratios close to 1 across all fractions; the spatial information on organellar origin (as shown in Figure 2B) will thus be lost. Using the individual SILAC channel intensities as a workaround to construct the organellar origin map has too low resolution. Regrettably, this is therefore not an option.

7. Fig 3B: why are some of the profiles so difference in the left and right panels? I believe these are supposed to indicate replicates?
As explained for point 5, the distribution within the 10K pellet is indeed variable especially in the HIV-infected condition. The profiles we chose to show do not hide this variability for the "green" proteins, but despite this variability of the overall green profiles between replicates, the 3 green proteins matched each other in both cases, thus showing robustness of the profiling approach.

8. Fig S3b and page 9: A change in fractionation pattern of a given protein does not necessarily mean that it associates with different EV subtypes, it may also mean that the size/shape/density of the EV itself is altered.
This is true. We have modified the sentence following description of Appendix fig S2 (formerly fig S3), p10 (formerly p9).
"*Changes in the fractionation patterns of the 26 candidates suggested either a change in their association with different EV subtypes, or a change of the size, shape and/or density of the EVs themselves (although in this case, multiple proteins should undergo the exact same shift).*"

9. Authors describe that MOV10 and SPN move to the virion in infected cells, however a biological role is not discussed or investigated.
This is true, but since our article is already very long, and submitted as a Resource article, we decided not to include experiments of inhibition of MOV10 and SPN expression (which were in a previous version of our manuscript: http://dx.doi.org/10.2139/ssrn.3516102), but focused exclusively on SERINC3 as a proof of concept follow up.

Referee #2:

The manuscript describes the protein composition of EVs from control and HIV infected cells. The article contains proteomic data that is better suited for specialized journals. The study lacks biological insights and is preliminary for EMBO J. The study also lacks any in vivo evidence of the claim "re-routed to non-viral EVs". Though the observations may be useful, the results are preliminary and lack biological/functional data.
This reviewer does not take into account the different requirements for a Resource article, which the Editors of EMBO Journal acknowledged as appropriate for our work. S/He also does not provide any hint as to what could be done to improve our article. We are thus sorry about his/her negative evaluation of our work, but we cannot answer his/her comments.

Referee #3:

In their manuscripts, "Proteomic profiling allows unbiased analysis of HIV-1 and house extracellular vesicles" Martin-Jaular and colleagues employed the SILAC quantitative proteomic profiling approach to analyze the composition of different extracellular vesicle (EV) fractions from Jurkat cell cultures. Using this technique they also analyzed EV compositions after infection with HIV. Their goal was to identify networks of closely interacting proteins (through the neighborhood network predictor) that would distinguish different types of EV and potentially reveal functional differences that could give insights into the role of pEV in general and HIV-induced EV specifically. The SILAC technology is well established and has been previously used to analyze compositions of vesicular structures of the cell, as for example Clathrin-coated vesicles.

While the technical approach of the manuscript is sound, the results in this lengthy and not easy to follow manuscript are rather disappointing and do not provide much of a new or meaningful insight. The authors state in their discussion: "to date, the majority of proteomic studies have provided a crude list of proteins found in both preparations of EV containing various mixtures of subtypes". While the authors indeed provide more than a crude list, their bioinformatic plots do not give a decisively better insight. Potetially, their vesicle fractionation based on different centrifugation steps (F1-F3) is not sophisticated enough to

match the SILAC approach In other words, this approach still creates of lot of overlap between the different types of vesicles and secretion pathways, particularly in the F3 pellet.
We cannot deny that there is still a lot of overlap of different EVs in the F3 pellet, but the added value is the profiling between the 3 fractions: this is clearly shown in figure S2A for CD63 and CD81, which display subtly different profiles even though both strongly enriched in F3, and resulting different neighbourhoods (figure 2). These results demonstrate that our approach provides a "decisively better insight", since classical proteomic analyses of the 100K or F3 fraction only would not have identified these differences between CD63 and CD81.

On the other hand the SILAC approach may not be sensitive enough (see below). Although we learn that the cellular proteins MOV10 and SPN are found in virus particles and that SERINC3 is shuttled into EV in the presence of Nef, the meaning of these findings remains unresolved. It is at least questionable that HIV uses a whole accessory gene to direct SERIN3 into an EV pathway in order to get rid of a restriction factor. Most likely there is a much bigger picture, at least with respect to Nef-induced EV secretion.
It is a common occurrence that restriction factors are counteracted by a viral protein. For instance, viral Vpu antagonizes the host restriction factor tetherin (Neil 2008, PMID: 18200009) and viral Vif induces the degradation of APOBEC3 (Marin 2003, PMID: 14528301). Moreover, it has been proposed that APOBEC can be packed into EVs/exosomes (Khatua 2009, PMID: 18987139). Our suggestion that Nef directs SERINC3 to EVs as a way of counteracting a restriction factor is in line with this idea. While there may be a 'bigger picture' of the functional consequences, this should not be required for a Resource article.

A main problem is a discrepancy with previous findings. Many proteins, particularly signaling proteins, Src kinases and proteases that have previously been identified in EV from HIV patients, HIV primary cell culture models and other in vitro systems were not identified in this analysis.
There is no discrepancy with previous findings generated by other groups with similar experimental systems (i.e. models of T lymphocytes infected by HIV-1). Proteins such as Src kinase, ADAM10 protease are present in our proteomics, with a possibly different set of neighbours in the control vs infected situation. They did not pass the restrictive threshold we choose to select candidate proteins for further follow up, thus maybe are not as strongly modified by HIV infection than the ones we selected. Our data set can be used for someone interested in a particular protein to get an idea of the extent of modification (and a low level of modification may still be interesting!). Conversely, our approach identified novel proteins not listed before as associated with virus infection (see table 2).

This raises suspicion that either the SILAC approach was not sensitive enough, or the cell culture model with Jurkat cells was not sufficiently representing the in vivo situation. In view of this discrepancy, and in order to validate their approach, the authors should have at least analyzed vesicles from primary T cell cultures and compare these results with the content in plasma extracellular vesicles from HIV patients.
As explained in the paragraph above, sensitivity of the SILAC method and the modification (MR plot) allows to identify novel proteins, and does not disqualify other previous studies performed with different biochemical approaches. Since our manuscript is already very long, we did not include additional mention of other studies performed with different models.
As for the model issue: Jurkat cells are CD4 T lymphoma cells, and an accepted model for primary CD4 T cells infection by HIV. We consider it at least as good as other models used

routinely in the literature but that do not have any characteristics of CD4 T cells (such as HEK293 or HeLa cells).
Following this reviewer's suggestion, however, in this revised version we have now analysed EVs from primary CD4+ T cells, using a range of biochemical approaches to explore the validity of the results obtained in Jurkat cells by the proteomic profiling approach. Note that proteomic profiling cannot be implemented on EVs from primary T cells since the approach requires rigorous quantification only achieved upon SILAC-labeling of the EV producing cells, which requires at least 5 full rounds of cell duplication. Primary CD4+ T cells only divide up to 4 times in vitro, upon activation. Nonetheless, these new experiments (new figure EV2) confirm similar co-localization data for the CD63, CD81 and CD3 proteins in EVs released by primary T cells, as in EVs released by Jurkat. The MACSPlexExo sandwich EV-capture approach shown in new Figure 3B-C (Jurkat) and new Figure EV2B-C (primary CD4+ T cells) shows preferential association of some proteins with either CD3 or CD63 in both Jurkat and primary cells (eg CD45 = PTPRC, with CD3-EVs), consistent with the NNP predictions.
We did not, however, perform in vitro infection of primary human CD4+ T cells by HIV-1, and characterize their EVs for presence of CD63, CD81, SERINC3, SPN, MOV10, because we know from our own previous work (M Ostrowski, K Witwer) that infection of activated primary CD4+ T cells leads to a high level of cell death, which makes separation of EVs from dead cell-derived vesicles very tricky. Uncontrolled cell death would introduce a strong variability, which will prevent reliable conclusions from such experiments.
For the other suggestion: since EVs in plasma from HIV patients are more likely to come from other cell types than CD4 T cells (as regularly indicated by results from the group of A.S. Baur: for instance Lee et al, EBiomed 2016, and 2018), analyzing such plasma would not provide any relevant information to validate our study.

Analyzing only the content of vesicles preparations may not provide the answers the field is hoping to find. It probably takes detailed molecular biology in conjunction with imaging, experimental marker proteins, sophisticated purification techniques and electron microscopy to identify different pathways of vesicle secretion including respective factor contents. For example there is evidence, that vesicle secretion in HIV infection is mediated by the so-called nonconventional secretion pathway (Zhao et al., 2019 Traffic), induced by HIV and mediated by a src kinase. None of the results presented here would point in such a direction. Therefore one has to question whether the here described approach and tool is sufficient to address these complex but state of the art questions.
We agree that our study does not specifically address different secretion mechanisms. However, this is a Resource article: we offer a conceptually unique and information-rich database for the EV field, as the basis for diverse follow up work.
The article quoted by this reviewer uses HEK293 cells as a model: our results in Jurkat cells do not contradict such results obtained in a completely different cell type (vesicle secretion is strongly influenced by endogenous characteristics of each cell type).

***

The Editor also shared with us cross-comments of reviewers 1 and 3, which were in line with their main comments we answered above.

One of the points re-raised is the apparent discrepancy with results from other groups, using different sources of EVs. In particular plasma EVs from HIV-infected patients are suggested. As explained above, such EVs are described not to originate primarily from CD4 T cells, thus our results cannot be compared with them, nor do they invalidate them.

Another main point is about the EV purification protocols, that would not be sufficient. However, this is probably a mis-understanding since we do not claim any superiority of our "purification" approach. Our approach is based not on the attempt to purify (as we and others had tried to do until now, with limited success), but on the quantitative profiling of proteins in EVs, without trying to obtain absolute purification of a single EV subtype (a most likely unreachable goal!). Other suggested EV isolation approaches, like SEC, are routinely used in the team, but not to purify better EVs, since these technics copurify all EV subtypes (we have recently published a snapshot on EVs where we highlighted the respective recovery/specificity properties of various isolation approaches: Cocozza et al, Cell 2020, PMID: *32649878)*

We hope that reviewer 1, following his/her original appreciation, can be convinced that the methodological concept and the results go beyond a half-way success, and that our comments on the other reviewer's points clarify the advances provided by our paper.

1st Revision - Editorial Decision 21st Dec 2020

Dear Clotilde,

Thank you for submitting your revised manuscript (EMBOJ-2020-105492R) to The EMBO Journal. Please accept my sincere apologies for getting back to you with this unusual protraction due to delayed reviewer input as well as detailed discussions here in the team. Your amended study was sent back to referees #1 and #3 for re-evaluation, and we have received comments from both, which I enclose below.

As you will see, referee #3 remains overall critical on the work. However, referee #1 stated that the issues raised have been comprehensively resolved and this expert is now broadly in favour of publication.

As detailed before we consider the core impact of your analysis as a conceptual methods advance and data set resource for the field. We appreciate the orthogonal experimentation including the biochemical validation and introduction of primary T cells as sufficient to solidify and corroborate these core aspects.

Thus, we are pleased to inform you that your manuscript has been accepted for publication in The EMBO Journal.

We need you to take care of a number of points related to formatting and data representation as detailed below, which should be addressed at re-submission.

Further, I will share additional changes and comments from our production team during the next days to be considered.

Please contact me at any time if you have additional questions related to below points.

As you might have noticed, every paper at the EMBO Journal now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal.
I look forward to your adjusted manuscript files.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Introduce ORCID IDs for all corresponding authors (G.B.) via our online manuscript system. Please see below for additional information.

>> Please adjust the title of your 'Declaration of Interests' statement to 'Conflict of Interest'.

>> Limit the number of keywords to maximally five, and the abstract to 175 words.

>> Release the privacy from your PRIDE data set and remove the token from the 'Data Availability' section.

>> Provide a separate 'Statistical Analysis' section detailing the algorithms applied.

>> Adjust the reference format to EMBO Journal style, limiting to 10 authors et al. .

>> Structured Methods format: Methods & Protocols section should come after Discussion; Reagents and Tools table should be uploaded as a separate file. Please move the Material and Methods part up after the Discussion.

>> Dataset EV titles and legends should be removed from manuscript and added to the EV tables and datasets.

>>Improve the resolution of Western blot data where possible.

****

Please note that as of January 2016, our new EMBO Press policy asks for corresponding authors to link to their ORCID iDs. You can read about the change under "Authorship Guidelines" in the Guide to Authors here: http://emboj.embopress.org/authorguide

In order to link your ORCID iD to your account in our manuscript tracking system, please do the following:

1. Click the 'Modify Profile' link at the bottom of your homepage in our system.
2. On the next page you will see a box half-way down the page titled ORCID*. Below this box is red text reading 'To Register/Link to ORCID, click here'. Please follow that link: you will be taken to ORCID where you can log in to your account (or create an account if you don't have one)
3. You will then be asked to authorise Wiley to access your ORCID information. Once you have approved the linking, you will be brought back to our manuscript system.

We regret that we cannot do this linking on your behalf for security reasons. We also cannot add your ORCID iD number manually to our system because there is no way for us to authenticate this iD number with ORCID.

Thank you very much in advance.

****

Referee #1:

The authors have appropriately addressed my concerns, and the additional experiments have strengthened the manuscript. I therefore recommend acceptance of this exciting work for publication.

Referee #3:

Upon a second closer look at the paper of Martin-Jaular and colleagues my initial skepticism remains or even got stronger. In the following I will summarize my concerns.

1. The text is much too long and too difficult to follow. With the revision this problem rather increased, meaning the text got even longer.

2. The lack of novelty is disappointing and troublesome. I see only little advance over our current knowledge. Even if this manuscript is scheduled as a resource paper, how could it be a resource if the initial results are not producing new meaningful insights? I personally would not know how I could use this resource for my research.

3. The effects that are described are mostly marginal and overstated or wrongly interpreted. For example:

In the abstract the authors claim:
"We then compared EVs from control and HIV-1-infected cells. HIV altered EV profiles of several cellular proteins, including MOV10 and SPN, which became specifically incorporated into HIV virions".
In order to claim that MOV10 and SPN were specifically incorporated into virions, the authors should have specifically isolated virions with anti-HIV specific antibodies (or using similar means) and then analyze the content. Otherwise the putative incorporation of MOV10 and SPN could also come from a co-migrating vesicle population (Figure 6).

They further claim in the abstract:
"SERINC3, which was re-routed to non-viral EVs in a Nef-dependent manner".
This conclusion is not correct. In their blots in figure 5C, SERINC3 runs in exactly the same non-virion fraction, whether the cells were infected or not. Hence the routing of this protein into EV seems to be not affected by the infection. Furthermore and importantly, there is no anti-NEF Western blot, which is necessary to proof a Nef dependent mechanism.

They further claim in the abstract:
"Furthermore, we found that SERINC3 controls the surface composition of EVs".

These results are really marginal being in the range of 0.5 to 0.2 fold of the control (Figure 6G). Since they were obtained with SERINC3 knockdown cells, off target effects cannot be excluded. Hence, a second independent method should have been employed to confirm these findings.

Another major finding of the manuscript is the specific incorporation of SPN into HIV virions in SERINC3 knockdown cells (figure 6C). They state in the text (page 13) :
"Although the amount of p24 is not affected by SERINC3 down-regulation, we detected an increase in the amount of SPN incorporation into sEVs/virus after HIV-1 infection."
The blot in Fig 6C shows one additional fraction that is positive for SERINC3. However, there is also one additional fraction positive for HIV p24. Hence there is no increase in SPN incorporation but simply a broader distribution of HIV particles in this particular gradient. Supporting this conclusion there is an almost perfect correlation between the amount of p24 and SPN protein in the same fractions.

4. In their revision the authors made not enough efforts to reconcile previous findings, particularly findings that were made in vivo or with primary cells and with respect to the alternative secretion pathway. They now describe that ADAM10 is also present in HIV induced vesicles. However, ADAM10 expression is low in T cells and not induced by HIV infection (which explains why it was not readily detected). For a valuable resource paper, the authors should somehow find a link to the published work done with primary material. In their response the corresponding author points out that SERINC3 has been described as relevant for HIV infection. Firstly, these were also pure in vitro results and secondly, a relevance of SERINC3 in vesicles in the pathogenesis of HIV infection has not been demonstrated. In fact, as the authors show, the protein is shuttle into vesicles in the presence or absence of the infection.

2nd Authors' Response to Reviewers 25th Jan 2021

The authors performed the requested changes.

2nd Revision - Editorial Decision 26th Jan 2021

Dear Clotilde Théry, dear Georg Borner,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper.

Also in case you might NOT want the transparent process file published at all, you will also need to inform us via email immediately. More information is available here:
http://emboj.embopress.org/about#Transparent_Process

Please note that in order to be able to start the production process, our publisher will need and contact you regarding the following forms:

- PAGE CHARGE AUTHORISATION (For Articles and Resources)
http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1460-2075/homepage/tej_apc.pdf

- LICENCE TO PUBLISH (for non-Open Access)

Your article cannot be published until the publisher has received the appropriate signed license agreement. Once your article has been received by Wiley for production you will receive an email from Wiley's Author Services system, which will ask you to log in and will present them with the appropriate license for completion.

- LICENCE TO PUBLISH for OPEN ACCESS papers

Authors of accepted peer-reviewed original research articles may choose to pay a fee in order for their published article to be made freely accessible to all online immediately upon publication. The EMBO Open fee is fixed at $5,200 (+ VAT where applicable).

We offer two licenses for Open Access papers, CC-BY and CC-BY-NC-ND.
For more information on these licenses, please visit: http://creativecommons.org/licenses/by/3.0/ and http://creativecommons.org/licenses/by-nc-nd/3.0/deed.en_US

- PAYMENT FOR OPEN ACCESS papers

You also need to complete our payment system for Open Access articles. Please follow this link and select EMBO Journal from the drop down list and then complete the payment process:
https://authorservices.wiley.com/bauthor/onlineopen_order.asp

Notably, please be reminded that under the DEAL agreement of European scientific institutions with our publisher Wiley, you could be eligible for free publication of your article in the open access format. Please contact either the administration at your institution or Wiley (embojournal@wiley.com) to clarify further questions.

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author(s) of the study. Please see the following link for representative examples: https://www.embopress.org/video_synopses

Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal
EMBO
Postfach 1022-40
Meyerhofstrasse 1
D-69117 Heidelberg
contact@embojournal.org
Submit at: http://emboj.msubmit.net

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: Georg H. H. Borner , Clotilde Théry
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2020-105492

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size has not been chosen.
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.	NA
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.	NA
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.	For some analysis, non-parametric tests were used that do not rely on distribution assumptions
Is there an estimate of variation within each group of data?	All samples are shown

USEFUL LINKS FOR COMPLETING THIS FORM

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

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

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

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).	Brand, catalog number and clone information are stated for all antibodies used.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	This information is included in the method section

* 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.	This study was conducted according to the Helsinki Declaration, with informed consent obtained from the blood donors, as requested by Institut Curie Review Board.
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.	This study was conducted according to the Helsinki Declaration, with informed consent obtained from the blood donors, as requested by Institut Curie Review Board.
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	Data availability The datasets and computer code produced in this study are available in the following databases: •Raw data : PRIDE PXD016626 (username: reviewer40036@ebi.ac.ukpassword: U7T0hcwK).
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.	Data availability The datasets and computer code produced in this study are available in the following databases: •Raw data : PRIDE PXD016626 (username: reviewer40036@ebi.ac.ukpassword: U7T0hcwK).

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.	NA