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MICAL2PV suppresses the formation of tunneling nanotubes and modulates mitochondrial trafficking

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1st Editorial Decision 7th Dec 2020

Dear Prof. Wu,

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and I think all their points need to be addressed, I will not detail them here.

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

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs

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http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in the respective section (e.g. 'No primary datasets have been generated and deposited'), see below.

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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 & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets 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])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal 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: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
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Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please find instructions on how to link the ORCID ID to the account in our manuscript tracking system in our Author guidelines:
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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

The manuscript by Wang et al. titled "MICAL2PV suppresses the formation of tunneling nanotubes and chemo-resistance of lung cancer cells" explores the hypothesis of the role of MICAL2PV in TNTs formation and mitochondria transfer. The data showing that enhanced mitochondrial infiltration following MICAL2PV down-regulation is dependent on F-actin are very nice. However, the approaches that they used to validate the hypothesis and the lack controls make the results quite speculative and they need to be sustained by additional experiments to grant publication. In addition, the results showing that the down-regulating MICAL2PV reduces chemo-induced cell death of lung cancer cells are not supporting the hypothesis.

Major comments:

1. Down-regulating MICAL2PV promotes TNT-mediated mitochondrial transfer between non-adjacent cells. The authors do not provide functional analysis of TNTs, nor quantification of the transfer or any live imaging assessing transfer. They only show a quantification of TNTs containing mitochondria by microscopy analysis. In my opinion this is not enough to support their hypothesis.Therefore the above outlined experiments will be required.

2. "Large cargoes, such as mitochondria, can only be transported via thick TNTs (> 0.7 μm in diameter) that containing microtubules (Dupont et al.,2018; Onfelt et al, 2006)". This is not correct, as we and other have observed mitochondria transfer in TNTs devoid of microtubules.

3. The concept that MICAL2PVs interacts with Miro2 and suppresses its mitochondrial localization, and the immunoprecipitation experiments supporting this are very nice, but then again additional data on the effect on the transfer of mitochondria are needed to support their conclusion that this affect mitochondria transfer.

4. Down-regulating MICAL2PV reduces chemo-induced cell death of lung cancer cells. This is the most problematic result of all. The co-culture experiments using donor-cells (healthy cells) expressing mito-mCherry with non -labelled acceptor-cells pretreated with chemotherapeutic drugs are not well presented. The authors should explain carefully the assay, what drug they use for the pre-treatment, how-long, and also if this drug has a reversible effect and for how long they can track the effectiveness of the drug because these parameters are essential for the conclusion. Also, they have no controls in this assay. They should use different labelling in order to discriminate the acceptor population. And they cannot conclude that the transferred mitochondria are healthy because mito-mcherry labels all the mitochondria of the donor population not only the healthy mitochondria.

5. Furthermore, to conclude that this is of any relevance for cancer they should use more relevant cancer models.

Minor comments:

1. "Consistent with the notion that MICAL2 plays an important role in lung cancer are our observation that reduced MICAL2 expression is correlated with decreased survival in multiple cohorts of patients". They should provide an immunohistochemistry staining for MICAL2PVa or MICAL2PVb (because there are not specific antibodies to discern between the two isoforms) of normal mucosa adjacent to lung cancer tissues showing the immunostaining in non-neoplastic lung tissue and the loss of staining in the metastasis region, not only the Kaplan-Meier graph.

Referee #2:

Wang et al. examine a spliced isoform of the neuronal guidance gene MICAL2 and its role as a potential biomarker that suppresses formation of tunnelling nanotubes (TNTs) in lung cancer cells. This particular set of isoforms, known as MICAL2PVa and Vb (referred to together as MICAL2PV in the manuscript), have been discovered and evaluated in prostate and lung tumors. Here, the authors describe that use of short hairpin RNA to down regulate this isoform leads to increase in intercellular communication among chemo-resistant and nondrug treated cells via TNTs, and the formation of TNTs is associated with increased intercellular transport of mitochondria. The central premise is that MICAL2PV provides a potential mechanistic explanation for regulation of TNTs in these cell lines, and that there is broader implication for cancer cell metabolism based on differences in mitochondrial transport between cells.

Assessment: The authors have performed extensive and very thought-provoking work, and are to be congratulated for their efforts. They build upon prior findings and discoveries in the field of tunnelling nanotube/TNTs biology, much of which is coming more to light just in the last few years, particularly with potential in vivo relevance in glioblastoma, lung cancers, mesotheliomas, ovarian cancers, and other invasive types of cancers. Rigorous experiments are performed to support strong claims that MICAL2PV is both necessary and sufficient in relation to suppressing TNT formation and related function in terms of mitochondrial transport.

My critiques and questions are as follows:
1. There is a heavy reliance on the use of the actin destabilizing agent Cytochalasin to attempt to abrogate TNTs through disruption of filamentous actin, thus demonstrating as a negative control for comparison results of assays, presuming TNTs are not present. Caution should be taken as this is not at all a TNT specific agent, as its action is very nonspecific. With this drug, as with Latrunculin (despite widespread use in the literature in relation to all forms of actin-based protrusions), cells are highly sensitive to either drug at many concentrations, which can also lead to decreased cell adherence which would naturally alter interpretation and accurate assessment of any cell protrusions, including TNTs.
2. The discussion in the second paragraph of introduction regarding cytonemes and MT-nanotubes is superfluous, as the focus of experimentation in this paper is clearly on TNTs predicated on F-actin, with wider variants more dependent on microtubules. There is also less justification for comparison as those other studies were performed in Drosophila, whereas the experiments here are clearly focused on more relevance to human cells.
3. In the latter half of the manuscript, there is emphasis on cells that are stressed through application of chemotherapeutic drugs, and comparison of observation to untreated healthy cells. Greater clarity should be made about how and in what duration the cells were exposed to chemotherapy; it is also not clear what the effect of chemotherapy drugs are on altering TNTs in these particular cells as well, independent of MICAL2PV. Several publications in the past few years (Desir et al 2018 Sci Rep; Tischchenko et al 2020 Cancers; and others) have supported the idea that chemo drugs themselves can alter TNTs in cancer cells.
4. In the second section of results, there is discussion of MICAL2 enhancing the process of EMT. However, this needs to be reconciled with its proposed simultaneous negative correlation of TNTs, as EMT has been associated with increased formation of TNTs in similar cells, including mesothelioma (referenced elsewhere in the manuscript for Methods, Lou et al. PLoS One 2012).
5. Although this may not be avoidable due to lack of specific antibodies for each variant,

interpretation of results must be taken into consideration that the results of the two isoforms Va and Vb are pooled rather than able to differentiate which one, if either, plays more of the "necessary and sufficient" role of MICAL2PV in suppressing TNTs in the cells.
6. Probably the most concerning experimental approach, in relation to conclusions made from the data, in the section entitled "Down-regulating MICAL2PV reduces chemo -induced cell death of lung cancer cells." Here, the authors coculture to groups of cells, and again use Cyto B to prove a point that abrogation of TNTs leads to a negative result. Nonetheless, the authors make extremely strong conclusions that the intercellular transfer occurred definitively via TNTs. It is not possible to make such a strong presumption, unless you also account for other possible modes of transfer, notwithstanding cell fusion, extracellular vesicles, other modalities, or any combination thereof that could explain intercellular transfer mitochondria that is seen here. Again also, per comment #3, were additional experienced performed to determine any potential effects of the chemotherapeutic drug on TNT quantities, independent of use of shM2?
7. In the subsequent section, the conclusion is that MICAL2PV "is both necessary and sufficient for suppressing TNT formation in lung cancer cells." The percent of reduction needs to be stated more clearly in the paragraph, and results explained more objectively. By description, use of the word "responsible" and otherwise imply hundred percent or near complete reduction, but this is not what is seen in the corresponding graphs. It is harder to reconcile the data with the notion that MICAL2PV is truly what one can consider "necessary and sufficient" to the degree espoused here.
8. Earlier in the manuscript, there is description of the initial finding of the isoforms of MICAL2PV in tumors presumably derived from human patients with prostate cancer and lung cancer. In the discussion, and in the appendix, there is reference to detection of mutations in the MO domain identified in patients as well. What would elevate the impact of this research would be to image the same tumors and demonstrate the same negative correlation of MICAL2PV isoforms to presence of TNTs, thus supporting in vivo relevance aside from the two cell lines used to derive the in vitro data.

Referee #3:

Wang et al., characterize the function of MICAL2V, an isoform of neural guidance MICAL2 as a suppressor of tunneling nanotubes (TNT) formation in a lung cancer model. Their data suggest that down-regulating MICAL2PV increases TNT formation and promotes intercellular transport of mitochondria and enhances survival of chemo-drug treated cells. In addition, they identify the monooxygenase (MO) domain of MICAL2PV as the mediator of TNT inhibition. The authors suggest that MICAL2PV is a regulator of mitochondrial trafficking. This is an interesting concept and supporting experiments are overall provided. However some of the conclusions are based on relatively preliminary data and need to be solidified. Other experiments need better explanations. My specific comments are below:

1) Two lung cancer cell lines (H1299 and A549 cells) were used. The reason for these choices needs to be explained.
2) Figure 1 analysis of TNT relies on markers of F-actin. The authors should provide TNT specific marker to validate their results. "These results clearly demonstrate the formation of TNT", these results despite the authors claim do not seem clear. The authors should also show quantification of these results. Are there differences in MICAL2PV isoform transcript expression (since antibody specific expression analysis is not possible)? The authors should make this clear.
3) In Fig 2 the authors show that reducing MICAL2PV levels increases TNT formation. What about over expression of MICAL2PV?
4) Figure 3 examines TNT transportation of mitochondria. The data is unclear. The authors should

use additional approaches to imaging to solidify their data. The authors should also provide additional data supporting the infiltration claim.
5) Experiments in Figs 5A and 5B are confusing, need better explanations.
6) It is unclear whether overexpression in experiments of Figs 6 and 7 are in stressed or healthy cells as the authors argued in their initial experiments that given the low TNT formation, they needed to stress the cells. In general the authors should explain how lung cancer cells are not already stressed and how additional stress for TNT formation may be physiologically relevant.
7) The authors do not explain why the focused on MICAL2 in lung cancer. Since MICAL2 expression was not detected in lung cancer samples, how is the Kaplan Meier data in Figure 5F explained? Additional information about how this data was generated would make the data more convincing.

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Editorial Board
EMBO Reports manuscript #EMBOR-2020-52006V1

February 6, 2021

Dear Editors:

We would like to submit our revised manuscript titled "**MICAL2PV suppresses the formation of tunneling nanotubes and modulates mitochondrial trafficking in lung cancer cells"** for consideration of publication in ***EMBO Reports***.

We greatly appreciate the constructive comments from all reviewers that helped us in improving our manuscript. We are grateful to reviewers who recognized that "extensive and rigorous experiments are performed" and that our "interesting concept" is supported by our data. We have carefully revised the manuscript following suggestions by the reviewers.

The following are the point-to-point responses to the comments by the reviewers.

Referee #1:

Overall response: we have taken the reviewer's suggestions, added more experiments, included more controls and removed speculative sentences.

Major comments:

1. Down-regulating MICAL2PV promotes TNT-mediated mitochondrial transfer between non-adjacent cells. The authors do not provide functional analysis of TNTs, nor quantification of the transfer or any live imaging assessing transfer. They only show a quantification of TNTs containing mitochondria by microscopy analysis. In my opinion this is not enough to support their hypothesis. Therefore the above outlined experiments will be required.

Response: we have taken the reviewer's suggestion and included a significant amount of new data. We have added live imaging data in four supplementary movies to demonstrate both intracellular mitochondrial trafficking and TNT-mediated mitochondrial transport. Time-lapse confocal microscopy using lung cancer cells expressing Mito-mCherry shows mitochondrial movement inside TNTs connecting non-drug treated and drug-treated cells (see Fig 6E and supplementary movie 1). However, it is difficult to quantify movement speed or number of mitochondria transported inside TNTs because of the transient and unstable nature of TNTs, even with our extensive expertise in live imaging and quantifying axonal mitochondrial transport (e.g., Chen et al, 2016). Down-regulating MICAL2PV significantly increased the proportion of TNTs that contain mitochondria and enhanced mitochondrial infiltration into the cortical cytoskeleton. Furthermore, we included data on TNT formation when different MICAL2PV mutants were expressed. In addition, our biochemical experiments demonstrate that MICAL2PV interacts with Miro2 and that down-regulating MICAL2 increases mitochondrial Miro2 levels. We further mapped domains in MICAL2PV and Miro2 involved in MICAL2PV-Miro2 interaction (Fig 4). Finally, knocking down Miro2 reverses the effect of MICAL2PV down-regulation in promoting mitochondrial infiltration into the cortical cytoskeleton and in increasing the formation of mitochondria-containing TNTs (Fig 5). Together, these data indicate that that MICAL2PV suppresses TNT formation and regulates intracellular mitochondrial trafficking/distribution. Our work uncovers the previously unknown role of MICAL2PV-Miro2 axis in regulating mitochondrial distribution.

2. "Large cargoes, such as mitochondria, can only be transported via thick TNTs (> 0.7 μm in diameter) that containing microtubules (Dupont et al.,2018; Onfelt et al, 2006)". This is not correct, as we and other have observed mitochondria transfer in TNTs devoid of microtubules.

Response: we have revised this part following reviewer's suggestion.

3. The concept that MICAL2PVs interacts with Miro2 and suppresses its mitochondrial localization, and the immunoprecipitation experiments supporting this are very nice, but then again additional data on the effect on the transfer of mitochondria are needed to support their conclusion that this affect mitochondria transfer.
Response: We thank the reviewer for the comment. We have revised the manuscript to properly address the issue of mitochondrial transport. We have carried out further experiments and included the data in the revised manuscript. First, we generated a panel of Miro2 mutants and mapped the Miro2-MICAL2PV interaction domain in Miro2. Our data indicate that the C-terminal domain of Miro2 (that is known to be important for mitochondrial localization of Miro2) is required for MICAL2PV-Miro2 interaction. Second, we examined whether MICAL2PV affected Miro2 expression and mitochondrial localization. Down-regulating MICAL2PV increases the level of the Miro2 protein associated with mitochondria. Consistently, decreased Miro2 mitochondrial localization was observed in cells transfected with MICAL2PV. These data suggest that MICAL2PV may inhibit the mitochondrial localization of Miro2 by interacting with its C-terminal domain. Furthermore, we performed knock-down experiments using specific Miro2 siRNAs and compared the mitochondrial subcellular distribution and formation of mitochondria-containing TNTs in control and shM2 (shMICAL2PV) lung cancer cells. These experiments show that down-regulating Miro2 significantly inhibited the mitochondrial infiltration in shM2 cells and reduced the proportion of TNTs containing mitochondria. These data reveal a role of the MICAL2PV-Miro2 axis in MICAL2PV function in lung cancer cells.

4. Down-regulating MICAL2PV reduces chemo-induced cell death of lung cancer cells. This is the most problematic result of all. The co-culture experiments using donor-cells (healthy cells) expressing mito-mCherry with non-labelled acceptor-cells pretreated with chemotherapeutic drugs are not well presented. The authors should explain carefully the assay, what drug they use for the pre-treatment, how-long, and also if this drug has a reversible effect and for how long they can track the effectiveness of the drug because these parameters are essential for the conclusion. Also, they have no controls in this assay. They should use different labelling in order to discriminate the acceptor population. And they cannot conclude that the transferred mitochondria are healthy because mito-mcherry labels all the mitochondria of the donor population not only the healthy mitochondria.
Response: We have taken the suggestion and revised the manuscript to clearly present the data. Detailed information, including the concentrations and duration of drug treatment, is now included both in the main text and in the method section, although it was only in the method section in the previous version. We have included proper controls in each set of experiment, for example, shCtr versus shM2 (i.e., shMICAL2PV) or vehicle control versus chemo-drug treatment, or the wild type MO domain versus the FAD-mutation containing MO domain.
We have also taken the reviewer's suggestion and included live cell imaging data in which co-culture experiments were carried out using healthy (non-drug pretreated) cells expressing mito-mCherry cells co-cultured with chemo-drug treated cells expressing mito-GFP (see Figure 6 and supplementary Movie 1). We agree that not all mitochondria transported are healthy, and we have revised the manuscript accordingly. Finally, we have included additional live cell imaging data to examine mitochondrial intracellular trafficking in control, shM2 and siMiro2/shM2 cells (see Supplementary movies 2-4). Our data demonstrate that knocking down Miro2 reversed the effect of MICAL2PV down-regulation on mitochondrial intracellular trafficking and formation of mitochondria containing TNTs. Also see more details in response to point 1.

5. Furthermore, to conclude that this is of any relevance for cancer they should use more relevant cancer models.
Response: We have screened a number of lung cancer cell lines for TNT formation, including A549, H23, H460, H520, H1299, H1993, and H1792. Initial experiments indicate that A549 and H1299 cells showed consistent results, whereas the heterogeneity in cell morphology and growth features making it difficult to consistently image and quantify TNTs in the other cell lines. A549 (K-Ras mutant) and H1299 (TP53-deficient) cells also represent lung cancer cell lines containing frequent genetic changes in lung cancer patients. For these reasons, these two lines were chosen for most experiments presented in this study. We agree that it is most desirable if TNT formation could be observed in solid tumor samples in vivo, which would then allow us to examine TNT formation in animal models or patient tissue samples. At the present, there is no technique that has been developed to detect TNT formation in solid tumor tissues, either post-mortem or in vivo. Such technical difficulties prevented us from using animal models or patient tissue samples in studying TNT formation.

Minor comments:

1. "Consistent with the notion that MICAL2 plays an important role in lung cancer are our observation that reduced MICAL2 expression is correlated with decreased survival in multiple cohorts of patients". They should provide an

immunohistochemistry staining for MICAL2PVa or MICAL2PVb (because there are not specific antibodies to discern between the two isoforms) of normal mucosa adjacent to lung cancer tissues showing the immunostaining in non-neoplastic lung tissue and the loss of staining in the metastasis region, not only the Kaplan-Meier graph.
Response: We appreciate the suggestion. Indeed, we made efforts to generate antibodies for detecting MICAL2PV protein in immunohistochemical staining. However, we have not been able to obtain an anti-MICAL2 antibody suitable for immunohistochemistry nor for differentiating MICAL2PVa vs MICAL2PVb isoforms, after spending several years testing anti-MICAL2 from 3 different vendors and also generating our customer produced antibodies, although we have obtained antibodies for Western blotting. We will continue to make efforts to generate better antibodies for immunohistochemistry in the future study.

Referee #2:
Overall response: we are grateful to this reviewer for the enthusiastic comments on our "extensive" and "rigorous experiments". We have taken the reviewer's suggestions and revised our manuscript accordingly
1. There is a heavy reliance on the use of the actin destabilizing agent Cytochalasin to attempt to abrogate TNTs through disruption of filamentous actin, thus demonstrating as a negative control for comparison results of assays, presuming TNTs are not present. Caution should be taken as this is not at all a TNT specific agent, as its action is very nonspecific. With this drug, as with Latrunculin (despite widespread use in the literature in relation to all forms of actin-based protrusions), cells are highly sensitive to either drug at many concentrations, which can also lead to decreased cell adherence which would naturally alter interpretation and accurate assessment of any cell protrusions, including TNTs.

Response: we are grateful to the reviewer's careful and thorough reading of our manuscript. Indeed, we were concerned over potential non-specific toxicity of these chemicals. Therefore, we decided to not only generate a series of deletion mutants of MICAL2PV that lack the MO domain but also make a mutant containing FAD-region specific point mutation that has been reported to be defective in actin-depolymerizing activity. Experiments using different MICAL2PV mutants that lack the actin-depolymerizing activity in comparison with either wild type MICAL2PV or wild type MO domain only gave us confidence in concluding that the MO domain and actin polymerizing activity are important for MICAL2PV in suppressing TNT formation. At the same time, we have carefully examined effects of both Cytochalasin B (Cyto B, to depolymerize F-actin) and Nocodazole (Noc; to depolymerize microtubules) at different concentrations. We have used appropriate concentrations for these reagents to minimize non-specific effects and included proper controls, such as the vehicle control (DMSO) and shCtr in addition to experiments using different mutants. At the concentration of 500 nM for 24hrs, which was used for all experiments shown, Cyto B did not affect cell morphology nor show any detectable cytotoxic effects. We have included proper discussion regarding the role of MO domain or actin-depolymerizing activity of MICAL2PV in TNT formation or mitochondrial distribution.

2. The discussion in the second paragraph of introduction regarding cytonemes and MT-nanotubes is superfluous, as the focus of experimentation in this paper is clearly on TNTs predicated on F-actin, with wider variants more dependent on microtubules. There is also less justification for comparison as those other studies were performed in Drosophila, whereas the experiments here are clearly focused on more relevance to human cells.

Response: We have taken the reviewer's suggestion and deleted superfluous part about Cytonemes and MT-nanotubes in the introduction and focused on the relevant issues.

3. In the latter half of the manuscript, there is emphasis on cells that are stressed through application of chemotherapeutic drugs, and comparison of observation to untreated healthy cells. Greater clarity should be made about how and in what duration the cells were exposed to chemotherapy; it is also not clear what the effect of chemotherapy drugs are on altering TNTs in these particular cells as well, independent of MICAL2PV. Several publications in the past few years (Desir et al 2018 Sci Rep; Tischchenko et al 2020 Cancers; and others) have supported the idea that chemo drugs themselves can alter TNTs in cancer cells.

Response: We have taken the suggestion and included detailed information on concentration and duration of drug treatment in the main text and in the method section (also see the response to reviewer 1). Indeed, we also observed that chemo-drug treatment also affected TNT formation (e.g. Fig 6B), consistent with the published studies. Other mechanisms may also contribute to chemo-resistance. In this study, we focused on characterizing effects of MICAL2PV in TNT formation and. We have included these points in the discussion section and added related references.
4. In the second section of results, there is discussion of MICAL2 enhancing the process of EMT. However, this needs to be reconciled with its proposed simultaneous negative correlation of TNTs, as EMT has been associated with increased

formation of TNTs in similar cells, including mesothelioma (referenced elsewhere in the manuscript for Methods, Lou et al. PLoS One 2012).
Response: We have revised the manuscript to clarify this issue. Although MICAL2 was reported to promote EMT in gastric cancer and renal cancer (Mariotti *et al*, 2016). We have not detected such effects in lung cancer. In fact, down-regulating MICAL2PV seemed to promote EMT in lung cancer cell (unpublished observation). Because this manuscript focuses on the role of MICAL2 in TNT formation and the issue of EMT requires extensive future work to resolve in a separate study, we will not include any data on EMT in this manuscript.

5. Although this may not be avoidable due to lack of specific antibodies for each variant, interpretation of results must be taken into consideration that the results of the two isoforms Va and Vb are pooled rather than able to differentiate which one, if either, plays more of the "necessary and sufficient" role of MICAL2PV in suppressing TNTs in the cells.
Response: It is likely that MICAL2PVa and MICAL2PVb have similar function in suppressing TNT formation because both isoforms share all predicted functional domains, including the MO, CH, LIM domain and the C-terminus mitochondrial targeting sequence (MTS); and the two isoforms only differ slightly in the sequence between the LIM domain and the MTS region. Our data indicate that the MO domain shows similar activity as the full-length MICAL2PVb in suppressing the TNT formation. Because of the lack of antibodies that distinguish MICAL2PVa from MICAL2Vb, we used MICAL2PV to include both isoforms. We have included more discussion to make it clear in the revised manuscript.

6. Probably the most concerning experimental approach, in relation to conclusions made from the data, in the section entitled "Down-regulating MICAL2PV reduces chemo -induced cell death of lung cancer cells." Here, the authors coculture to groups of cells, and again use Cyto B to prove a point that abrogation of TNTs leads to a negative result. Nonetheless, the authors make extremely strong conclusions that the intercellular transfer occurred definitively via TNTs. It is not possible to make such a strong presumption, unless you also account for other possible modes of transfer, notwithstanding cell fusion, extracellular vesicles, other modalities, or any combination thereof that could explain intercellular transfer mitochondria that is seen here. Again also, per comment #3, were additional experienced performed to determine any potential effects of the chemotherapeutic drug on TNT quantities, independent of use of shM2?
Response: We have taken the reviewer's suggestion and revised the manuscript accordingly. We have removed "intercellular mitochondrial transfer" in the manuscript where appropriate. In this study, we have focused on the role of MICAL2PV. Therefore, the effect of chemo-drug 5-FU was examined in comparison to vehicle control or by comparing control vs shM2 cells. Also see response to comment#3. We agree that other modes of mitochondrial transport could contribute and have revised those sentences.

7. In the subsequent section, the conclusion is that MICAL2PV "is both necessary and sufficient for suppressing TNT formation in lung cancer cells." The percent of reduction needs to be stated more clearly in the paragraph, and results explained more objectively. By description, use of the word "responsible" and otherwise imply hundred percent or near complete reduction, but this is not what is seen in the corresponding graphs. It is harder to reconcile the data with the notion that MICAL2PV is truly what one can consider "necessary and sufficient" to the degree espoused here.
Response: We have removed the word "responsible" here and changed "both necessary and sufficient" in revised the description following the reviewer's suggestion. In the revised manuscript, we have included the changes in the percentage of cells forming TNTs after expression of different MICAL2 mutants.

8. Earlier in the manuscript, there is description of the initial finding of the isoforms of MICAL2PV in tumors presumably derived from human patients with prostate cancer and lung cancer. In the discussion, and in the appendix, there is reference to detection of mutations in the MO domain identified in patients as well. What would elevate the impact of this research would be to image the same tumors and demonstrate the same negative correlation of MICAL2PV isoforms to presence of TNTs, thus supporting in vivo relevance aside from the two cell lines used to derive the in vitro data.
Response: We agree with this reviewer that it would elevate the impact of the work if TNTs could be imaged in tumor tissues or in vivo. Unfortunately, the current technology does not allow imaging of TNTs in tumor tissues. Also see response to reviewer #1. Future studies are clearly necessary to overcome the technical challenges in imaging TNTs in the compact environment as in the tumor samples.

Referee #3:
Overall response: we are grateful to this reviewer for providing constructive comments and for stating that "This is an interesting concept and supporting experiments are overall provided".

1) Two lung cancer cell lines (H1299 and A549 cells) were used. The reason for these choices needs to be explained.
Response: We have taken the reviewer's suggestion and included the rationale in the revised manuscript. Briefly, H1299 and A549 lung cancer cell lines are used because they represent the most common types of lung cancer in patients, with H1299 representing TP53-deficient NSCLC and A549 represent Kras mutant (G12S), the most common genetic mutations in human lung cancer. In addition, we have tested a number other lung cancer cell lines available from ATCC, including H1993, H23, H460, H520 and H1792. However, the heterogeneity in cell morphology and cellular features making it difficult to clearly quantify TNTs in other cell types.

2) Figure 1 analysis of TNT relies on markers of F-actin. The authors should provide TNT specific marker to validate their results. "These results clearly demonstrate the formation of TNT", these results despite the authors claim do not seem clear. The authors should also show quantification of these results. Are there differences in MICAL2PV isoform transcript expression (since antibody specific expression analysis is not possible)? The authors should make this clear.
Response: We agree with the reviewer that it would be ideal to have TNT-specific biomarkers. Unfortunately, no specific markers for TNTs have been identified. Therefore, the published studies on TNTs are based on three characteristics that are adopted in our study: 1) they connect at least two cells; 2) they do not attach to the substrate, distinguishing them from other cellular protrusions, such as filopodia; 3) they contain F-actin. F-actin is the fundamental component for TNTs. The quantification of two types of TNTs was measured in shCtr and shM2 cells in supplementary figure 3 (approximately 80% of TNTs contained microtubule).
Regarding MICAL2PV isoforms, both MICAL2PVa and MICAL2PVb are detected in normal lung tissues and in lung cancer samples. Because our shRNAs were designed to target both MICAL2PVa and MICAL2PVb isoforms in their shared regions. In this study, we did not differentiate MICAL2PVa from MICAL2PVb because these isoforms share most domains and we have not seen any differences in all assays examined. We have added more description and quantification to make it clearer in the revised manuscript.

3) In Fig 2 the authors show that reducing MICAL2PV levels increases TNT formation. What about over expression of MICAL2PV?
Response: In the supplementary figure S2 of the revised manuscript, we included the experiments of overexpressing MICAL2PV on TNT formation. The spontaneous rate of TNT formation was low, so we induced TNT formation by stress using reported method (Ady *et al*, 2014). Our data show that overexpressing MICAL2PV significantly inhibited the stress-induced TNT formation. These data have been incorporated into Figure 2 and Figure2 EV in the revised paper.

4) Figure 3 examines TNT transportation of mitochondria. The data is unclear. The authors should use additional approaches to imaging to solidify their data. The authors should also provide additional data supporting the infiltration claim.
Response: We have taken the reviewer's suggestion and included additional experiments, including live cell imaging of mitochondrial transport along TNTs and of intracellular mitochondrial trafficking. For details, see response to reviewer #1.

5) Experiments in Figs 5A and 5B are confusing, need better explanations.
Response: We have taken the reviewer's suggestion and revised the manuscript. Figure 5 in the previous version has been reorganized with added new data, including an image from live cell confocal imaging demonstrating mitochondria being transported toward the chemo-drug treated cells. Also see response to reviewer #1.

6) It is unclear whether overexpression in experiments of Figs 6 and 7 are in stressed or healthy cells as the authors argued in their initial experiments that given the low TNT formation, they needed to stress the cells. In general, the authors should explain how lung cancer cells are not already stressed and how additional stress for TNT formation may be physiologically relevant.
Response: Indeed, the rate of TNT formation was low in cells cultured under conventional conditions. In order to more clearly detect the effect of MICAL2PV overexpression on TNT formation, we induced the formation of TNTs by using 2-DG and conditioned medium as previously reported. In Figures 6 and 7, however, shM2 cells were used for overexpressing MICAL2PV and different mutants. In shM2 cells the rate of TNT formation was high in the regular media, therefore, we used regular media. TNT formation can be detected in the lung cancer cells (~8% in H1299 and ~4% in A549) under the regular

culture conditions. When testing effects of MICAL2PV expression, we used either 2-DG or conditioned media to enhance TNT formation in order to clearly detect the effect of MICAL2PV in suppressing TNT formation. These culture conditions are relevant to tumor microenvironment in that rapid tumor growth often leads to nutritional deficiency and hypoxia. We have included more detailed description in the revised manuscript.

7) The authors do not explain why the focused on MICAL2 in lung cancer. Since MICAL2 expression was not detected in lung cancer samples, how is the Kaplan Meier data in Figure 5F explained? Additional information about how this data was generated would make the data more convincing.

Response: Previous studies have shown that MICAL2 was overexpressed in breast cancer, gastric cancer, and might play a role as an oncogene (Mariotti *et al.*, 2016; Wang *et al*, 2018). However, the role of MICAL2 in lung cancer was unclear. Our analyses of public datasets using Oncomine and other tools, indicate that the mRNA levels of MICAL2 were down-regulated in a large fraction of lung cancer patient samples and the low expression of MICAL2 is associated with poor prognosis. These observations suggest that MICAL2 may play a completely different role in lung cancer, distinct from its role in other types of cancer. Therefore, we decided to focus on investigating the role of MICAL2 in lung cancer. Although the full length MICAL2V1 was not detectable in patient lung cancer samples, there is still a range of expression levels for MICAL2PV among different patient samples, albeit lower than those in the normal lung tissues. To alleviate the concern, we have decided to remove this patient survival data from the manuscript.

All authors declare that there is no financial conflict of interest.

The following researchers in the field may serve expert reviews: Drs. Saghi Ghaffari (Icahn School of Medicine at Mount Sinai; an expert in cancer biology; Saghi.Ghaffari@mssm.edu), Philip W. Hinds (phinds@tuftsmedicalcenter.org, Phil.Hinds@tufts.edu; a leading scientist cancer research), Jin Chen (Vanderbilt Medical Center; an expert in cancer biology; jin.chen@Vanderbilt.Edu), Ramaswamy Govindan (an expert in lung cancer; Washington University; rgovinda@dom.wustl.edu), Dean G. Tang (Dean.Tang@Roswellpark.org; an expert in cancer biology) and Adam Marcus (Emory University; a well-known researcher in cancer invasion and metastasis; aimarcu@emory.edu).

Thank you very much for your consideration.

Sincerely yours,

Jane Wu, M.D., Ph.D.

Charles Louis Mix Professor of Neurology

1st Revision - Editorial Decision 24th Mar 2021

Dear Prof. Wu,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referee #3 now supports the publication of your study in EMBO reports. Nevertheless, referees #1 and #2 have remaining concerns and suggestions to improve the manuscript, we ask you to address in a final revised manuscript. Please note that we consider it as a prerequisite for publication here that intercellular mitochondrial trafficking through TNTs is demonstrated using the experimental approaches indicated by the referees. Please also provide a detailed point-by-point-response addressing the remaining concerns of referees #1 and #2.

Moreover, I have these editorial requests I also ask you to address in a final revised manuscript:

- Please shorten the title to not more than 100 characters.

- Please provide the abstract written in present tense.

- Please call the 'competing interest statement' 'Conflict of interest statement'.

- Please add page numbers and a table of contents (TOC) including page numbers to the Appendix.

- Please name the movie files Movie EVx and change their call outs accordingly. Please upload these ZIPed together with their legend as a text file. Finally, please remove the movie legends form the main manuscript text.

- Thank you for submitting the source data. Please submit the source data as one PDF file per figure (main and EV figures). Please submit the source data for Appendix figures as one pdf. Please remove the statement regarding source data from the Appendix. Please add respective notes to the images they apply to.

- Please also add a formal "Data Availability" section to the manuscript after the methods section. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated or deposited').

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me

know if you have questions regarding the revision.

Yours sincerely

Achim Breiling
Editor
EMBO Reports

Referee #1:

The Authors have partially replied to my comments, although they have made substantial efforts and the paper has improved. However, I do not agree that their movie (mov 1) shows transfer; it only shows active mitochondria movement in a TNT-like structure between cells. A pretty big problem in my view is that the authors never assessed mito transfer using co-culture assays and compared the contact-dependent transfer to secretion under their different conditions (controls that were requested). These kind of experiments and controls should be done. I agree that their data shows that by down regulating MICAL they see more mitochondria present in cell-to-cell connections and that mito positioning is different, but they make a big leap in my opinion that this means more transfer. So if accepted this needs to be rephrased.

Referee #1:
Major comments:
1. Down-regulating MICAL2PV promotes TNT-mediated mitochondrial transfer between non-adjacent cells. The authors do not provide functional analysis of TNTs, nor quantification of the transfer or any live imaging assessing transfer. They only show a quantification of TNTs containing mitochondria by microscopy analysis. In my opinion this is not enough to support their hypothesis. Therefore the above outlined experiments will be required.

Response: we have taken the reviewer's suggestion and included a significant amount of new data. We have added live imaging data in four supplementary movies to demonstrate both intracellular mitochondrial trafficking and TNT-mediated mitochondrial transport. Time-lapse confocal microscopy using lung cancer cells expressing Mito-mCherry shows mitochondrial movement inside TNTs connecting non-drug treated and drug-treated cells (see Fig 6E and supplementary movie 1). However, it is difficult to quantify movement speed or number of mitochondria transported inside TNTs because of the transient and unstable nature of TNTs, even with our extensive expertise in live imaging and quantifying axonal mitochondrial transport (e.g., Chen et al, 2016). Down-regulating MICAL2PV significantly increased the proportion of TNTs that contain mitochondria and enhanced mitochondrial infiltration into the cortical cytoskeleton. Furthermore, we included data on TNT formation when different MICAL2PV mutants were expressed. In addition, our biochemical experiments demonstrate that MICAL2PV interacts with Miro2 and that down-regulating MICAL2 increases mitochondrial Miro2 levels. We further mapped domains in MICAL2PV and Miro2 involved in MICAL2PV-Miro2 interaction (Fig 4). Finally, knocking down Miro2 reverses the effect of MICAL2PV down-regulation in promoting mitochondrial infiltration into the cortical cytoskeleton and increasing the formation of mitochondria-containing TNTs (Fig 5). Together, these data indicate that that MICAL2PV suppresses TNT formation and regulates intracellular mitochondrial trafficking/distribution. Our work uncovers the previously unknown role of MICAL2PV-Miro2 axis in regulating mitochondrial distribution.

Referee: I really appreciate the effort to do live-imaging to show the transfer of mitochondria from

donor-cell (mito-mCherry) to an acceptor cell (mito-GFP). The arrow and the movement of the two fluorophores are really nice, by the contrary the Brightfield image is static, so we cannot visualize the integrity of the TNT over time and the movement of the two cells, this information is really important because we need to know that the cells did not join together (decreasing the distance between the donor and acceptor), in order to exclude that the mitochondria trafficking from the donor cell is in reality a retraction effect where the mitochondrion is really coming back to the donor because of a retraction of the cell, often due to the laser effect. Also, it is important to show the integrity of the TNT during the movie. To address this all the time frames should have the three channels red green and brightfield.

However, it is difficult to quantify movement speed or number of mitochondria transported inside TNTs because of the transient and unstable nature of TNTs. About the speed of the transfer of mitochondria is true that is difficult to get a significant statistical number, but it could be useful to have an approximate idea of the duration of the transfer. Nonetheless it is required to quantify the amount of transfer. This is relatively simple as the authors can calculate the number of acceptor cells receiving mitochondria by FACS. Also I would prefer that the authors say "TNTs with mitochondria inside" as you cannot affirm that they are transporting when you are quantifying fixed samples.

2. "Large cargoes, such as mitochondria, can only be transported via thick TNTs (> 0.7 μm in diameter) that containing microtubules (Dupont et al.,2018; Onfelt et al, 2006)". This is not correct, as we and other have observed mitochondria transfer in TNTs devoid of microtubules.
Response: we have revised this part following reviewer's suggestion.

Referee: Thanks, you should still cite papers in which mitochondria have been seen transferring in thin TNTs containing only actin.

3. The concept that MICAL2PVs interacts with Miro2 and suppresses its mitochondrial localization, and the immunoprecipitation experiments supporting this are very nice, but then again additional data on the effect on the transfer of mitochondria are needed to support their conclusion that this affects mitochondria transfer.

Response: We thank the reviewer for the comment. We have revised the manuscript to properly address the issue of mitochondrial transport. We have carried out further experiments and included the data in the revised manuscript. First, we generated a panel of Miro2 mutants and mapped the Miro2-MICAL2PV interaction domain in Miro2. Our data indicate that the C-terminal domain of Miro2 (that is known to be important for mitochondrial localization of Miro2) is required for MICAL2PVMiro2 interaction. Second, we examined whether MICAL2PV affected Miro2 expression and mitochondrial localization. Downregulating MICAL2PV increases the level of the Miro2 protein associated with mitochondria. Consistently, decreased Miro2 mitochondrial localization was observed in cells transfected with MICAL2PV. These data suggest that MICAL2PV may inhibit the mitochondrial localization of Miro2 by interacting with its C-terminal domain. Furthermore, we performed knock-down experiments using specific Miro2 siRNAs and compared the mitochondrial subcellular distribution and formation of mitochondria-containing TNTs in control and shM2 (shMICAL2PV) lung cancer cells. These experiments show that downregulating Miro2 significantly inhibited the mitochondrial infiltration in shM2 cells and reduced the proportion of TNTs containing mitochondria. These data reveal a role of the MICAL2PV-Miro2 axis in MICAL2PV function in lung cancer cells.

Referee: The question was about the effect of the inhibition of the transfer of mitochondria, ei, if the cells that do not receive the mitochondria because the transfer of mitochondria is inhibited, are

more sensitive to chemotherapy treatment, enter in apoptosis, have less ability to invade, their metabolism is different to the control that receive mitochondria (more glycolysis instead of OXPHOs), or have less proliferative and invasiveness features. Again, they should quantify the number of acceptor cells receiving mitochondria and not only the TNTs with mitochondria inside, using FACs.

4. Down-regulating MICAL2PV reduces chemo-induced cell death of lung cancer cells. This is the most problematic result of all. The co-culture experiments using donor-cells (healthy cells) expressing mito-mCherry with non-labelled acceptor-cells pretreated with chemotherapeutic drugs are not well presented. The authors should explain carefully the assay, what drug they use for the pre-treatment, how-long, and also if this drug has a reversible effect and for how long they can track the effectiveness of the drug because these parameters are essential for the conclusion. Also, they have no controls in this assay. They should use different labelling in order to discriminate the acceptor population. And they cannot conclude that the transferred mitochondria are healthy because mito-mcherry labels all the mitochondria of the donor population not only the healthy mitochondria.

Response: We have taken the suggestion and revised the manuscript to clearly present the data. Detailed information, including the concentrations and duration of drug treatment, is now included both in the main text and in the method section, although it was only in the method section in the previous version. We have included proper controls in each set of experiment, for example, shCtr versus shM2 (i.e., shMICAL2PV) or vehicle control versus chemo-drug treatment, or the wild type MO domain versus the FAD-mutation containing MO domain. We have also taken the reviewer's suggestion and included live cell imaging data in which co-culture experiments were carried out using healthy (non-drug pretreated) cells expressing mito-mCherry cells co-cultured with chemo-drug treated cells expressing mito-GFP (see Figure 6 and supplementary Movie 1). We agree that not all mitochondria transported are healthy, and we have revised the manuscript accordingly. Finally, we have included additional live cell imaging data to examine mitochondrial intracellular trafficking in control, shM2 and siMiro2/shM2 cells (see Supplementary movies 2-4). Our data demonstrate that knocking down Miro2 reversed the effect of MICAL2PV down-regulation on mitochondrial intracellular trafficking and formation of mitochondria containing TNTs. Also see more details in response to point 1.

Referee: Thanks, now it is clearer.

We have included proper controls in each set of experiment, for example, shCtr versus shM2 (i.e., shMICAL2PV) or vehicle control versus chemo-drug treatment, or the wild type MO domain versus the FAD-mutation containing MO domain.

Referee: These controls are very good contribution, but maybe, we were not clear about what type of controls we were referring to. Referee 2 also has the same concern that us. We were speaking about controls to eliminate the possibility that the transfer occurs via secretion (exosomes ...). It is mandatory to eliminate this possibility.

We agree that not all mitochondria transported are healthy, and we have revised the manuscript accordingly. Mitochondrial intracellular trafficking in Supplementary movies 2-4.

Referee: This is a very good contribution, showing a pretty clear phenotype. However I think that they should perform more transfer analysis and add it to figure 6.

5. Furthermore, to conclude that this is of any relevance for cancer they should use more relevant cancer models.

Response: We have screened a number of lung cancer cell lines for TNT formation, including A549, H23, H460, H520, H1299, H1993, and H1792. Initial experiments indicate that A549 and H1299 cells showed consistent results, whereas the heterogeneity in cell morphology and growth features making it difficult to consistently image and quantify TNTs in the other cell lines. A549 (K-Ras mutant) and H1299 (TP53-deficient) cells also represent lung cancer cell lines containing frequent genetic changes in lung cancer patients. For these reasons, these two lines were chosen for most experiments presented in this study. We agree that it is most desirable if TNT formation could be observed in solid tumor samples in vivo, which would then allow us to examine TNT formation in animal models or patient tissue samples. At the present, there is no technique that has been developed to detect TNT formation in solid tumor tissues, either post-mortem or in vivo. Such technical difficulties prevented us from using animal models or patient tissue samples in studying TNT formation.

Referee: I am not sure about this answer, they could have used primary cells.

Referee: Some minor comments:
- Figure 2C: These brightfield images could be replaced by other fluorescence ones, in such a way that the cells and TNTs are better appreciated.
- "Therefore, we measured the percentage of TNTs transporting mitochondria in control and shM2 cells following immunofluorescent confocal microscopy": I think it would be better to change the word "transporting" to "containing"
- "suggesting that MICAL2PV may suppress TNT-mediated mitochondrial exchange between lung cancer cells": I do not agree with this statememt, since when they knock-down MICAL2PV they have more TNTs and more TNTs containing mitochondria, therefore the possible suppressive role does not have to be nec essarily on the transfer as they state....
- Videos are played very fast, should be slowed down
- Movie 1: these are cells treated with the chemodrug, therefore they should specify it and this movie should be part of the chemo-therapy paragraph.

Referee #2:

The authors have returned with a revised manuscript containing new data resulting from the suggestions of multiple reviewers. They have also made edits softening their conclusions. The lack of a specific antibodies for MICAL2PVa vs MICAL2PVb remains a shortcoming, acknowledging the difficulty of generating such specific antibodies against each epitope but at the same time much of the conclusions are predicated on work that make conclusions differentiating between the two. The supplementary movie 1 was a nice depiction of trafficking; the other movies did not substantially add to supporting the conclusions.

What came across with the initial submission and unfortunately again in portions of the response with this revised version is a general lack of insight or acknowledgement into state-of-the-art investigation already seen in the field of TNT biology. While investigation and characterization of TNTs in general is relatively new over the past 15 years, there has been a spike in excellent work done across the spectrum of cell types, in cancer and in non-cancer, over the past five years. The lack of insight is most apparent in the rebuttal surrounding intercellular mitochondrial trafficking, as the reviewer for the comment correctly mentions that many groups have established and validated

methods for studying this aspect of TNTs; another example comes in the statement "At the present, there is no technique that has been developed to detect TNT formation in solid tumor tissues, either post-mortem or in vivo. Such technical difficulties prevented us from using animal models or patient tissue samples in studying TNT formation." The statement in that first sentence is not true, and evidence can be readily found in a growing number of papers that over the past decade have reported use of confocal microscopy to visualize tunnelling nanotubes and tumour microtubes in tissue - in animals and in human tumors, ex vivo and in vivo - that could have been applied here. To state that no such technique or approach exists gives the impression that the authors have not appreciated the background of the field accomplished by other groups, and placing their work within the existing context of what is known. All of the reviewers stated concern about this and it does not appear the current response fully addressed these concerns adequately enough despite a larger body of data.

Referee #3:

The authors have adequately revised the manuscript and responded to my concerns. I think the manuscript is novel and of major interest specifically in exposing the mechanisms of mitochondrial trafficking and distribution by MICAL2.

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Dr. Achim Breiling
Editor
EMBO Reports manuscript #EMBOR-2020-52006V1

Apr 12, 2021

Dear Dr. Breiling:

We would like to resubmit this manuscript titled "**MICAL2PV suppresses the formation of tunneling nanotubes and modulates mitochondrial trafficking in lung cancer cells**".

We appreciate very much the constructive comments from all reviewers that helped us in improving our manuscript. We have taken reviewers' suggestions and addressed concerns raised. We have added new data and revised the manuscript accordingly.

The following are point-to-point responses to specific comments:
Reviewer 1
1. *The major concern is that TNT-mediated mitochondrial transfer should be assessed using co-culture followed by quantifying the number of acceptor cells receiving mitochondria by FACS to show cell-contact dependence and to exclude the* possible secretion mediated-transfer.
Response: We thank the reviewer for the suggestion.
We have carried out these experiments and added the data into the revised manuscript (Fig 3 and Fig 6). Using Mito-GFP labeled donor cells (shCtr or shM2) and mCherry recipient cells, we have carried out co-culture assay following published protocols (Abounit et al, 2015; Pinto et al, 2021). Following the reviewer's suggestion, we have included co-cultures in transwells in which donor and recipient cells were separated by a filter. Indeed, our data from such co-culture coupled FACS analyses demonstrate that down-regulating MICAL2PV promotes mitochondrial transfer (Figure 3) and that such mitochondrial transfer is dependent on cell contact and independent of secretion-related mechanisms. In our co-culture experiments, we also included a group in which culture media was switched to galactose media to examine whether such "galactose" stress might affect TNT-mediated mitochondrial transport. Interestingly, cells exhibited increased mitochondrial transport in the galactose group as compared with the cells in the glucose media. Furthermore, we also carried out co-culture-FACS analyses of shM2 cells when Miro2 was down-regulated and show that down-regulating Miro2 reduced mitochondrial transfer in shM2 cells (Figure 6F). These data support a role of the MICAL2PV-Miro2 axis in regulating TNT-mediated mitochondrial transfer in lung cancer cells.

2. Presentation of live cell images in all 3 channels and use "TNTs with mitochondria inside" to replace "transporting mitochondria"
Response: We taken the reviewer's suggestion to presented the time frames of the live imaging data in Fig 3A and also in supplementary Movie1 and Movie2
we have taken the reviewer's suggestion to use "TNTs containing mitochondria".
3. Effects of MICAL2PV inhibition on mitochondrial transfer should be assessed by the number of acceptor cells receiving mitochondria using FACS.
Response: We have taken the reviewer's suggestion and perform these experiments (see above).

4. Inclusion of controls to eliminate the possibility of mitochondrial transfer through secretion
Response: We have taken the reviewer's suggestion to include the requested controls (also see above) and excluded the possible secretion related mechanisms in mediating mitochondrial transfer that we observed.
In addition to data shown in Fig3, we have added a set of experiments to investigate whether secretion contributes to reduced cell death of chemo-drug treated shM2 cells in co-cultured assay (Figure 7B). We measured cell viability when drug-treated cells were cultured in the conditioned medium obtained from the untreated cells or co-cultured with untreated cells in transwells separately by a filter. When cultured in conditioned medium or co-cultured with untreated cells in transwells, these cells did not show changes in cell viability, indicating that the reduction in cell death of chemo-drug treated shM2 in the co-culture assay shown in Fig7A was cell contact dependent.

5. Add more "mitochondrial transfer" analysis to Figure 6.
Response: Because the percentage of cells receiving donor derived mitochondria was small even in relatively healthy control cells, this has made it very difficult to quantify mitochondrial transfer in 5-FU treated cells in which there was significant cell death. Therefore, we focused on examining the role of TNT formation in chemo-resistance. To avoid confusion, we removed the confocal imaging panel. In this study, we addressed the role of TNT formation in chemo-resistance and would like to address the issue of mitochondrial transfer in chemo-resistance in our future studies.

6. you should still cite papers in which mitochondria have been seen transferring in thin TNTs containing only actin.
Response: We have revised the manuscript according to reviewer's suggestion and cited relevant published studies.
Minor comments:
-Replace the bright field images in Figure 2C by fluorescence ones.
Response: The images in Figure 2 were taken in living cells without fluorescence staining. However, we have included a fluorescence figure in supplementary Figure S2 in which TNTs were marked by white arrows.
-Change "the percentage of TNTs transporting mitochondria" to "the percentage of TNTs containing mitochondria"
Response: We taken the suggestion and made corresponding changes.
-Revise the statement to "suggesting that MICAL2PV may suppress TNT formation and mitochondria-containing TNTs"
Response: We taken the suggestion and made corresponding changes.

- Videos are played very fast, should be slowed down
Response: We taken the suggestion and made corresponding changes.

- Movie 1: these are cells treated with the chemodrug, therefore they should specify it and this movie should be part of the chemo-therapy paragraph.
Response: As described in question 5. We have replaced the videos to illustrate that mitochondria transport along TNTs in shCtr and shM2 cells (Movie 1 and Movie 2).

Reviewer 2
1. Lack of specific antibodies differentiating MICAL2PVa and MICAL2PVb.
Response: Because both MICAL2PVa and MICAL2PVb are highly similar in protein sequences, and both these isoforms are expressed in lung tissue and cancer cells, there is no strong rationale for us to differentiate MICAL2PVa and MICAL2PVb isoforms. In fact, in all experiments done so far, MICAL2PVa and MICAL2PVb behaved in a very similar, if not identical, manner. As a result, we did not make efforts to differentiate these two isoforms. This referee might have confused MICAL2PV with MICAL2V1 isoform, which is not expressed in lung tissue or cancer cells.

2. The reviewer felt that we did not adequately cite published studies.
Response: We thank this reviewer for the suggestion. We have revised the manuscript according to reviewer's suggestion and more clearly cited relevant published studies.

It should be noted that our study focuses on the role of MICAL2PV in TNT formation and mitochondrial trafficking, including intracellular mitochondrial trafficking. Down-regulating MICAL2PV significantly increased the proportion of TNTs that contain mitochondria and enhanced mitochondrial infiltration into the cortical cytoskeleton. Data from the co-culture experiments support that down-regulating MICAL2PV increased TNT-mediated mitochondrial transfer. We have demonstrated that the MO domain of MICAL2PV is required for suppression of TNT formation. In addition, our biochemical experiments demonstrate that MICAL2PV interacts with Miro2 and that down-regulating MICAL2 increases mitochondrial Miro2 levels. We

further mapped domains in MICAL2PV and Miro2 involved in MICAL2PV-Miro2 interaction. Finally, knocking down Miro2 reverses the effect of MICAL2PV down-regulation in promoting mitochondrial infiltration into the cortical cytoskeleton and in increasing the formation of mitochondria-containing TNTs. Together, these data indicate that that MICAL2PV suppresses TNT formation and regulates intracellular mitochondrial trafficking/distribution. Our work uncovers a previously unknown role of the MICAL2PV-Miro2 axis in regulating mitochondrial distribution and transport.

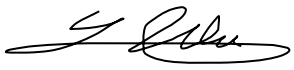
We thank the reviewer 3 for her/his comments. As pointed out by reviewer 3, our discovery of the role of MICAL2PV in TNT formation and mitochondrial trafficking is “novel and of major interest”.

We sincerely hope that the significance of our study is appreciated and that you find our revision and response satisfactory.

All authors declare that there is no financial conflict of interest.

Thank you very much for your consideration.

Sincerely yours,



Jane Wu, M.D., Ph.D.

Charles Louis Mix Professor of Neurology

2nd Revision - Editorial Decision 30th Apr 2021

Dear Prof. Wu

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the report from the referee that was asked to re-evaluate your study, you will find below. As you will see, the referee now supports the publication of your study in EMBO reports. Nevertheless, s/he has 2 remaining points I ask you to address in a further revised version of the manuscript. Please also provide a final point-by-point response regarding the remaining concerns of the referee.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Kind regards,

Achim Breiling
Editor
EMBO Reports

Referee #1:

The authors have performed the experiments suggested, in particular regarding the co-culture experiments using two distinct populations as donors and acceptor cells.

However figure 3G is of poor quality and should be replaced by a nice panel(as in fig 1C or 1D) where the two cell populations are readily distinguishable.

Further, it would be necessary to show at least one example (if not more) of TNT connection of donor and acceptor cells.

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Dr. Achim Breiling
Editor
EMBO Reports manuscript #EMBOR-2020-52006V1

May 3, 2021

Dear Dr. Breiling:

We would like to resubmit this manuscript titled "**MICAL2PV suppresses the formation of tunneling nanotubes and modulates mitochondrial trafficking**".

We have taken the reviewer's suggestions and addressed concerns raised. We have added new data and revised the manuscript accordingly.

The following are point-to-point responses to specific comments:
Reviewer 1
The authors have performed the experiments suggested, in particular regarding the co-culture experiments using two distinct populations as donors and acceptor cells. However figure 3G is of poor quality and should be replaced by a nice panel(as in fig 1C or 1D) where the two cell populations are readily distinguishable.

Response: We thank the reviewer for the suggestion.
We have now updated Figure 3G in which the donor and recipient cell populations are easily distinguishable. The experiment clearly demonstrated the transfer of mitochondria from the donor cell to the recipient cell.

Further, it would be necessary to show at least one example (if not more) of TNT connection of donor and acceptor cells.

Response: We have added a movie of live cell confocal imaging that demonstrates mitochondrial movement inside the TNT connecting a Mito-GFP labeled donor cell and a mCherry labeled recipient cell.

We sincerely hope that you find our revision and response satisfactory.

All authors declare that there is no financial conflict of interest.

Thank you very much for your consideration.

Sincerely yours,

Jane Wu, M.D., Ph.D.

Charles Louis Mix Professor of Neurology

3rd Revision - Editorial Decision 7th May 2021

Prof. Jane Wu
Northwestern University Feinberg School of Medicine
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Dear Prof. Wu,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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

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

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

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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:Jane Y. Wu
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2020-52006V1

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?	NA
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.	Yes. Whenever possible, data analyses were performed by researchers blinded to sample IDs. In microscopic imaging analyses, images were taken from random fields without bias, instead of selecting fields with subjective bias. In addition, all cells photographed were included in the statistical analyses, rather than selecting a subset of cells for analyses. Furthermore, the results were confirmed by different researchers.
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.	Yes. For dataset small than 2000 elements, Shapiro-Wilk normality test was used to determine whether the data were normally distributed. When dataset containing more than 2000 elements, Kolmogorov-Smirnov test was used to determine whether the data were normally distributed.
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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://jij.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Anti-ATP5B antibody (WB and human, Proteintech Cat#17247-1-AP, RRID:AB_2061878, PMID: 30898010); Anti-KIF5B antibody (WB and human, Proteintech Cat#21632-1-AP, RRID:AB_11182931, PMID:30605689); Anti-Miro1 antibody (WB and human, Sigma-Aldrich Cat# WH0055288M1, RRID:AB_1843347, PMID:32280985); Anti-Miro2 antibody (WB and human, Proteintech Cat#11237-1-AP, RRID:AB_2179539, PMID:31602805); Anti-β-Tubulin antibody (WB/IF and human, Proteintech Cat#10094-1-AP, RRID:AB_2210695, PMID:31061090); Anti-β-Actin antibody (WB and human, Proteintech Cat#60008-1-Ig, RRID:AB_2289225, PMID:31989756, PMID:32376691); Anti-Tom20 antibody (IF and human, Proteintech Cat#11802-1-AP, RRID:AB_2207530, PMID:30527743, PMID:31101394, PMID:31793879); Anti-Flag antibody (WB/IP, Sigma-Aldrich Cat# F3165, RRID:AB_259529, PMID:18265009, PMID:18752272, PMID:23321641, PMID:23525242, PMID:24932806, PMID:25232112, PMID:25319695); Anti-GFP antibody (WB, Probes Cat# A-6455, RRID:AB_221570, PMID:10518537, PMID:10882121, PMID:10893270, PMID:10944533, PMID:10950951, PMID:11038191, PMID:11092884, PMID:11245583); Anti-HA antibody (IF/IP/WB, Abmart Cat# M20003, RRID:AB_2864345, PMID: 3047011); Anti-Myc antibody (WB/IF/IP, Proteintech Cat# 60003-2-Ig, RRID:AB_2734122, PMID:31593505); Alexa Fluor® 594 Phalloidin antibody (IF, Thermo Fisher Scientific Cat# A12381, RRID:AB_2315633, PMID:27966429, PMID:28193690, PMID:28244368, PMID:30021164, PMID:30067966, PMID:30098998); Goat Anti-Mouse IgG1 Antibody, Alexa Fluor 488 Conjugated (IF, Molecular Probes Cat# A-21121,
	RRID:AB_2535764, PMID:24174666, PMID:25485969, PMID:26969737, PMID:27338237, PMID:27381477, PMID:27725085); Goat Anti-Mouse IgG1 Antibody, Alexa Fluor® 594 Conjugated (IF, Molecular Probes Cat# A-21125, RRID:AB_141593, PMID:25194025, PMID:29882512); Alexa Fluor® 488 goat anti-rabbit IgG (IF, Thermo Fisher Scientific Cat# A-11008, RRID:AB_143165, PMID:10089887, PMID:10216085, PMID:10367906, PMID:10399916, PMID:10417176); Alexa Fluor® 594 goat anti-rabbit IgG (IF, Molecular Probes Cat# A-11012, RRID:AB_141359, PMID:24737644, PMID:25916839, PMID:26470600, PMID:26571427, PMID:28343982); Alexa Fluor® 647 goat anti-mouse (IF, Molecular Probes Cat# A-31571, RRID:AB_162542, PMID:15297669, PMID:15703277, PMID:18273885, PMID:19245833).
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 obtained from ATCC and authenticated by STR profiling. They are routingly tested for mycoplasma contamination.

* 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).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.