THE EMBO JOURNAL

NALCN-mediated sodium influx confers metastatic prostate cancer cell invasiveness

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Transaction Report:

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

1st Editorial Decision **7th Sep 2022**

Dear Dr Prevarskaya,

Thank you again for the submission of your manuscript (EMBOJ-2022-112198) to The EMBO Journal, and your patience with our decision at this time of the year. Your study has been sent to three reviewers with cancer and Ca2+ channel signaling expertise, however one reviewer got much delayed and has not sent us his-her report so far. We have received feedback from the other two referees, which I enclose below, and decided to - in the interest of the timeliness of the data - proceed with our decision based on these reports.

As you will see, the referees acknowledge the potential interest and novelty of your results, although they also express a number of major issues that will have to be conclusively addressed before they can be supportive of publication of your manuscript in The EMBO Journal. The reviewers raise a number of points related to additional experiments required to deepen the insights into NACLN's function in metastasis and and controls to consolidate the findings. They also point to amendments of the data discussion and literature references as well as the overall structure changes of the manuscript, that would need to be conclusively addressed to achieve the level of robustness and clarity needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments, pending there are no overriding technical concerns raised by referee #3.

I will share the report of this expert as soon as we receive it.

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

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

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

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

Instruction for the preparation of your revised manuscript:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response 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://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition).
In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.
*** Note - All links should resolve to a page where the data can be accessed. ***

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 .

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

10) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

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

11) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

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

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (6th Dec 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #1:

In their MS the authors identify a novel and important role for NALCN channel in mediating Na+ signaling, which in turn communicates with the cell membrane NCX mitochondrial NCLX and SERCA to initiate Ca2+ oscillations that trigger the secretory remodeling process required for invadopogenesis leading to tumorigenesis and metastasis in cell models and in vivo. This an important, interesting, and fundamental study that is well-executed, and written.
Several relatively minor issues should be, however addressed:
1) the size of the figure captions and illustration is small, and there are blurred, making it very difficult to see; the author should revise all the figures to increase the size of the caption and solve the blurring issues.
2) the author used siNCLX to control its expression they should determine by WB if they achieved effective silencing.
3) Similarly, they note the importance of mitochondrial Ca2+ signaling in their pathway they should by using Rhod2, for example or other mito Ca2+ reporters if their effects are linked to changes in mitochondrial Ca2+ signaling

3) the author suggests in their final scheme that Na+ taken up by NALCN is triggering mitochondrial Ca2+ signaling into the mitochondria, but they don't show it I am aware that this may be challenging due to the current lack of good mitochondrial Na+ dyes/ They should at least describe previous studies that relate to this and the link between Ca2+ ROS and SOC.

Referee #2:

In their current study the authors describe the role of the NALCN Na2+ channel in prostate cancer. To this end, they suggest that NACLN functionally interacts with Na2+/Ca2+ exchanger in the mitochondria and in the plasma membrane. Moreover, the authors indicate involvement of ROS, SOC channels and Src kinase in the NACLN-controlled regulatory axis that fuels cancer cell invasion.
This is an interesting work that has the potential to expand our understanding on how ion channels control cancer cell biology. I have several comments and suggestions:

Major:
- The coregulation between NALCN, SOC, NCX, NCLX and SERCA/IP3R needs a more mechanistic examination since their interplay is in the core of this study.

- The contribution of ROS needs further investigation. The authors could artificially manipulate the redox state i.e. induce mitochondrial ROS production and apply antioxidants to evaluate the role of ROS on NALCN-controlled cancer cell phenotype. An additional approach for measuring ROS in addition to mitoSOX would be beneficial.

- The data presentation can be improved and the manuscript could profit from a figure rearrangement. Following the main text and the red line of the study would thus be easier.

- The effects of NALCN manipulation on the therapeutic response of the prostate cancer cells should be evaluated.

Minor:
- The abstract needs to be more compact and should more clearly describe the main findings as well as checked for typos. See for example: Mechanistically, NALCN associates functionally.... Or ..... Na+ leak channel, NALCN, at the hot spots..... (should be hot spot)

- The authors should discuss the role of ROS and SOC channels in cancer in more detail and in light of their current findings. How is it possible that mitochondrial ROS affect plasma membrane channels if the antioxidant systems in the cytosol are functional?

- The IHC images shown in Fig. 1 need to be quantified and statistically tested. Moreover, it has to be confirmed that in Fig 1c the differences in expression levels are due to upregulation of NALCN in the cancer cells and not due to increased infiltration of other cell types such as immune cells.

- How was the metastatic potential of the cells shown in Fig 1e determined?

- Migration and invasion assays (see Fig. 1f) should be performed in at least one additional cell line

- The supplementary figures should be reorganized and ordered as they are mentioned in the text.

- Please provide WB-based quantification of the shRNA efficacy.

- Images presented in Fig 2a should be quantified.

- In Fig S2g only intracellular pH is measured. The text claims that both the extrusion and the intracellular pH was measured in

S2g

- The authors describe the involvement of several proteins (Src, Tsk5, Cdc42etc) in the NALCN-controlled signaling axis. However, the rationale as to why exactly these proteins were selected is not clear. The paper would profit for more clear explanation in this regard.

- Why was the Fura-2 signal normalized to 1? With ratiometric dyes this is not needed and wanted since the information about the resting calcium level is lost.

- All colocalization images should be quantified.

- Calcium channels have been reported to affect prostate cancer cell growth. However, the authors of this study do not observe such biological effects although calcium signaling is affected. The authors might want to comment on this.

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Daniel Klimmeck, PhD
Senior Scientific Editor
The EMBO Journal
Heidelberg, Germany

January 23, 2023

Dear Dr. Klimmeck,

Following your e-mail of 7th September 2022 we are now submitting the revised version of our manuscript *"NALCN-mediated sodium influx regulates metastatic prostate cancer cell invasiveness"* former tile *"Expression of neuronal NALCN provides for persistent invasion of metastasizing prostate cancer cells"*. We prefer to modify the title since central factor involved in the regulation of the cancer cell invasiveness is the function of NALCN channel (i.e. sodium influx) rather than it's expression the protein *per ce*. To clarify the main message of the manuscript, as it was requested by both reviewers, we have modified virtually all the figures, both main and supplementary, and produced an appendix with two additional figures. In the revised version of our manuscript, virtually all the images are enlarged and the results of additional data analysis are presented where it was requested. That was done for the cost of omission of some data presented in the previous version of our manuscript. The order of the authors in the list of authors was modified accordingly. Also, I would like to mention that all the revision and additional experiments have been done by Antoine Folcher and Dmitri Gordienko. Oksana Iamshanova (the third

author on the list) did not participate in the revision work and relative contribution of the results obtained by her is diminished in the revised manuscript.

Thank you for giving us an opportunity to respond to the critiques provided by the two Reviewers of our manuscript. We also thank both Reviewers for their time, attention, interesting comments and helpful suggestions.

We are very pleased that the novelty and importance of our findings was well recognized by both expert reviewers who noted that
Reviewer 1:" this is an important, interesting, and fundamental study that is well-executed and written"
Reviewer 2: " this is an interesting work that has the potential to expand our understanding on how ion channels control cancer cell biology".

The Reviewers also highlighted some important points that needed clarification. We have addressed each of these comments carefully and provide below a detailed response to their critiques. In particular, we have re-arranged presentation of our data, conducted additional analysis and, when it was necessary, additional experiments. These data and all requested revisions of the text and figures are now included in the revised submission.

Indeed, both reviewers asked us to improve the presentation of figures: ***"the size of the figure captions and illustrations is small"; "the data presentation can be improved and the manuscript could profit from a figure rearrangement. Following the main text and the red line of the study would thus be easier"***.
We agree with the reviewers on this general comment. To clarify the main message of the manuscript, we have modified virtually all the figures, both main and supplementary, and produced an appendix with two additional figures. In the revised version of our manuscript, virtually all the images are enlarged and the results of additional data analysis are presented where it was requested. That was done for the cost of omission of some data presented in the previous version of our manuscript. The text of the manuscript and the figure legends were modified in accordance with rules of the EMBO Journal.

Finally, you asked us to comment on the recent paper of Rahrmann et al, Nature Genetics, 2022. Indeed, it is important and interesting paper, however, any mechanistic insight on how NALCN could regulate dissemination has not been provided. In the new version of our manuscript we have included into Discussion the paragraph addressing this paper: *"Recently, it has been shown that the deletion of Nalcn from gastric adenocarcinomas in mice increased the number of circulating tumor cells and distant metastasis in mice (Rahrmann et al., 2022). The authors also identified a number of nonsynonymous mutations and, using HOLE analysis (Jackson et al., 2001) and predicted that most (but not all) of them in gastric and colorectal cancers as loss-of-function. Although this theoretical analysis provides an important background for further investigations, only functional studies of the mutated channel activity may deliver the information about significance of the selected mutations and unravel the molecular mechanisms linking loss of NALCN function to cell dissemination. It is now well established that oncochannelopathies could involve the same channel for distinct functions in different types of cancers and in a context dependent manner (for review see Prevarskaya et al., 2018). For example, one of the*

recent studies provides evidence that SOCE, in contrast to that reported previously (Sun et al., 2014), may also extinguish invasive behaviour by specific molecular mechanism dependent on cholesterol biosynthesis, and that the suppression of this mechanism promotes invasion and metastasis (Gross et al., 2022). Further studies are needed to understand the role of NALCN alterations in different types of human cancers."

We hope that in light of all these modifications you can now find our work worthy of publication in *EMBO Journal*.

Reply to Reviewer 1 comments:

The authors thank the Reviewer for critical comments which have helped us to improve our work. Below are point-by-point responses to the criticism raised.

Critique R1.1 : "*the size of the figure captions and illustration is small, and there are blurred, making it very difficult to see; the author should revise all the figures to increase the size of the caption and solve the blurring issues".*

Response R1.1: To clarify the main message of the manuscript, we have modified virtually all the figures, both main and supplementary, and produced an appendix with two additional figures. In the revised version of our manuscript, virtually all the images are enlarged and the results of additional data analysis are presented where it was requested. That was done for the cost of omission of some data presented in the previous version of our manuscript.

Critique R1.2: "*the author used siNCLX to control its expression they should determine by WB if they achieved effective silencing".*

Response R1.2: Indeed, in the previous version of the manuscript the siNCLX efficiency has been checked by qPCR quantification (previous Figure S7j). This transfection by siNCLX showed a decrease of 50% at the mRNA level. To address the reviewer request, we have done the western blotting and quantified the effect of this silencing at the protein level. The result is now presented in Appendix, Figure S1E.

Critique R1.3*: "Similarly, they note the importance of mitochondrial Ca2+ signaling in their pathway they should by using Rhod2, for example or other mito Ca2+ reporters if their effects are linked to changes in mitochondrial Ca2+ signaling."*

Response R1.3: We have directly demonstrated that pro-invasive factors (like FBS and EGF) induced Ca^{2+} oscillations not only in the cytoplasm but also in the ER and mitochondria (Figures 3 and 5). The ER origin of the Ca^{2+} events reported by Mag-Fluo-4 was confirmed by the expression of DsRed2-ER (Figure 3A). The mitochondrial origin of the Ca^{2+} events reported Rhod-2 was confirmed by double-staining with MitoTracker Green (Figure 5A). Furthermore, we have demonstrated that cytosolic [Ca^{2+}] oscillation reported by Fluo-4 were associated with oscillation of superoxide production by mitochondria reported by MitoSOX Red (Figure 5B).

Furthemore, to address the question of the Reviewer 2 – *"How is it possible that mitochondrial ROS affect plasma membrane channels if the antioxidant systems in the cytosol are functional?"* – we have analyzed spatial re-organization of mitochondria following stimulation with 10% FBS of PC-3 cells expressing AcGFP-Mito. We found that mitochondria started to accumulate at the sites of invadopodia formation (see Figure below panel A and video Mito_PM24frs.avi) thus creating micro domains where mitochondrial ROS may affect the activity of the channels/transporters in the cell plasma membrane. Formation of NALCN-enriched "rosettes" at these sites was demonstrated in separate experiments (see Figure below panel B and video NALCN_PM24frs.avi).

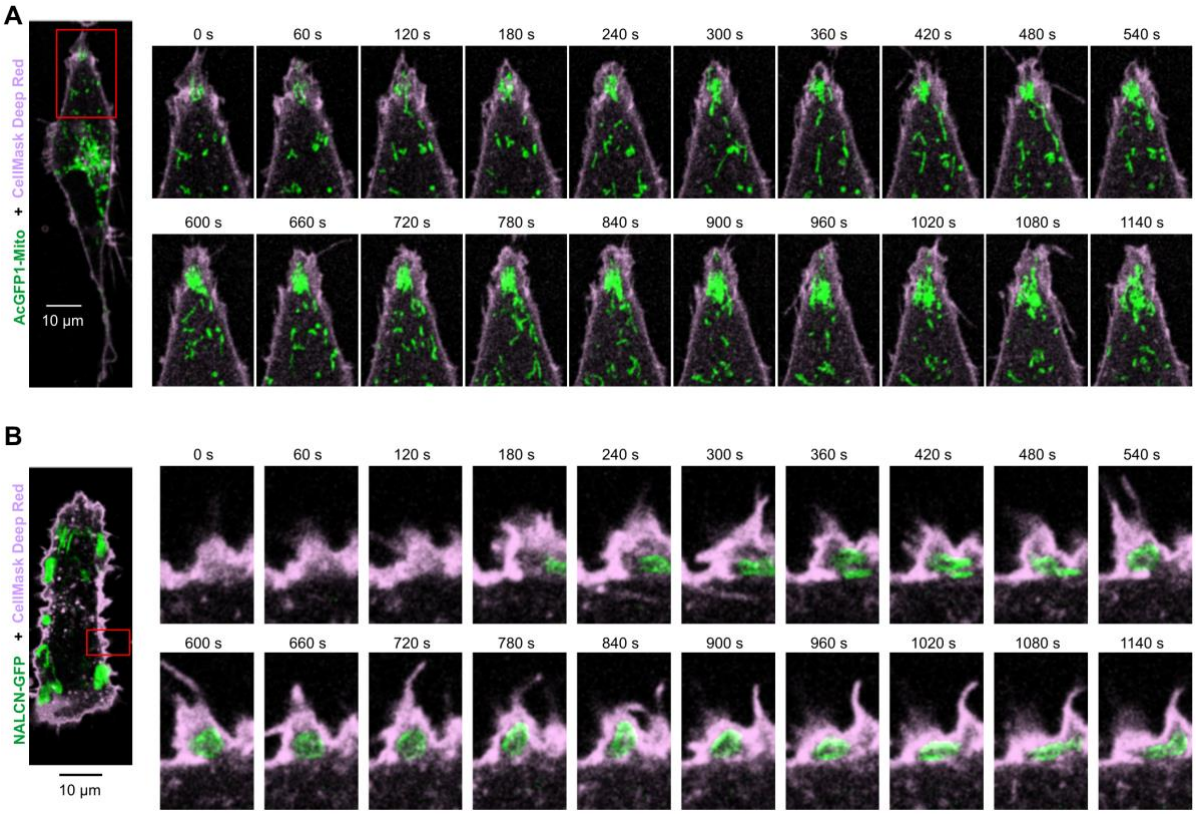


Figure. Accumulation of mitochondria and NALCN-enriched structures at the sites of invadopodia formation.
PC-3 cells expressing either AcGFP1-Mito (**A**) or NALCN-GFP (**B**) were stained with CellMask Deep Red. The x-y time series confocal imaging was performed at 0.1 Hz after stimulation of the cells with 10% FBS. The galleries (right) show every 6th image captured from the boxed regions (left). Note accumulation of mitochondria (**A**) and formation of NALCN-enriched "rosette" at the site of invadopodia formation. Please also see enclosed animations.

These data will be a part of separate article focused on regulation of plasmalemmal proteins by mitochondria that is currently in preparation.

Critique R1.4*: " the author suggests in their final scheme that Na+ taken up by NALCN is triggering mitochondrial Ca2+ signaling into the mitochondria, but they don't show it I am aware that this may be challenging due to the current lack of good mitochondrial Na+ dyes/ They should at least describe previous studies that relate to this and the link between Ca2+ ROS and SOC."*

Response R1.4: Indeed, to our knowledge, the current Na^+ mitochondrial dyes are not of sufficient quality and the results are not reliable.
Even though direct monitoring of Na^+ uptake into the mitochondria seems to be arduous, we have demonstrated the contribution of NCLX to Na^+/Ca^{2+} signaling in axis of prostate cancer cells using siRNA technology. Please see Figures 4H, 5D and S4C of the revised manuscript.

As requested by the reviewer we included into the discussion section (last paragraph) previous studies on Na+/Ca2+ mitochondrial feedback and the link between Ca^{2+}, ROS and SOC.

Reply to <u>Reviewer 2 comments.</u>

The authors thank the Reviewer for critical comments which have helped us to improve our work. Below are point-by-point responses to the criticism raised.

<u>Major comments</u>

Critique R2.1*: "The coregulation between NALCN, SOC, NCX, NCLX and SERCA/IP3R needs a more mechanistic examination since their interplay is in the core of this study."*

Response R2.1 In Figure 3 of the revised manuscript we have demonstrated that FBS induces calcium oscillations in the cytoplasm and ER of PC3 cells. These oscillations were drastically decreased after the silencing of NALCN or removal of extracellular Na^+. The mechanism triggering these oscillations is the release of Ca^{2+} from the ER mediated via IP_3Rs (see Figure below) and subsequent activation of Orai1/STIM1 pathway leading to SOCE.

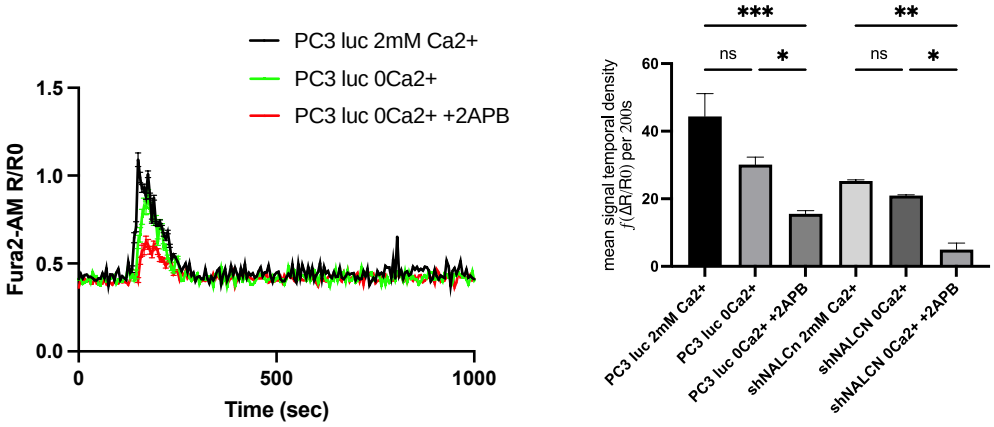


Figure. The effect of the removal of extracellular or inhibition of IP_3Rs with 2-APB on the FBS-induced $[Ca^{2+}]_c$ transient. Note that removal of extracellular Ca^{2+} prier to FBS application had no significant effect while inhibition of IP_3Rs significantly inhibited the FBS-induced $[Ca^{2+}]_c$ transient. Also note that these effects did not depend on NALCN bioavailability.

To further explore this process, we used Thapsigargin (TG), the irreversible inhibitor of the SERCA pump. As shown in Figure 4D, in Ca^{2+}-free extracellular medium, TG did not induce Na^+ entry. This proves the involvement of Ca^{2+} infux via store-operated channels (SOCs) in induction of in Na^+ influx mediated by NALCN (Figure 4I). The role of Orai and STIM1 in SOCE and Na^+ entry was confirmed using siRNA technology (Figure S4A).
Is functional NALCN, in turn, required for full-scale SOCE? This was assessed using shNALCN PC3 cells and revealed that NALCN suppression not only decreased initial SOCE amplitude but also accelerated its rate of decline (Figure 4E). On the one hand, the decrease of the SOCE amplitude by shNALCN suggests positive feedback between NALCN and SOCE, so that functional NALCN seems to be required for full-scale SOCE: activation. On the other hand, the acceleration of the rate of decline of SOCE was also caused by inhibition of the reverse-mode plasmalemmal Na^+/Ca^{2+} exchanger (RM-NCX) with KB-R7943 (Figure 4F and S4B), which was observed only in the presence of Na^+ in extracellular solution (Figure 4G). These results strongly suggest that NALCN-mediated Na^+ influx, in turn, engages Ca^{2+} influx via RM-NCX, what was confirmed further by the effect of KB-R7943 on cytosolic $[Ca^{2+}]$ oscillations (Figure 4J) and whole-cell inward current (Figure 4K). Thus, translation of Ca^{2+} into Na^+ signaling mediated by SOCE to NALCN coupling is converted back to Ca^{2+} oscillations via NALCN to RM-NCX coupling. One of the central elements tuning this conversion appeared to be mitochondrial Na^+/Ca^{2+} exchanger (NCLX). Indeed, similarly to inhibition of RM-NCX with KB-R7943, suppression of NCLX with siRNA (siNCLX) accelerated the rate of decline of SOCE (Figure 4H) and abrogated cytosolic $[Ca^{2+}]$ oscillations (Figure 5D). This tuning of cytosolic $[Ca^{2+}]$ oscillations by NCLX may involve direct recycling of Ca^{2+} by mitochondria (Figure 5A) and less direct effect of mitochondrial ROS (Figure 5B and C) on SERCA (also recruited to Ca^{2+} recycling; see Figure 3A) and SOCs..

Critique R2.2*: "The contribution of ROS needs further investigation. The authors could artificially manipulate the redox state i.e. induce mitochondrial ROS production and apply antioxidants to evaluate the role of ROS on NALCN-controlled cancer cell*

phenotype. An additional approach for measuring ROS in addition to mitoSOX would be beneficial."

Response R2.2: External application of 50μM H_2O_2 significantly attenuated cytosolic [Ca^{2+}] oscillations (Figure 5C). In accordance with you suggestion, to evaluate the effect of ROS on the prostate cancer cell invasiveness we conducted Boyden chamber – based invasion assays. We assessed the effects of (i) external application of 50 μM H_2O_2 and (ii) induction of mitochondrial ROS production by inhibition of mitochondrial electron transport chain complex III with 10 μM of Antimycin A. These experiments were conducted on the 2 groups of cells: control PC-3 cells (shCTRL) and shNALCN PC-3 cells (see Figure below). In both cell groups treatment with H_2O_2 dramatically attenuates the relative invasion rate. The effect of Antimycin A was weaker than that of 50 μM H_2O_2 and was attenuated (yet being significant) in shNALCN PC-3 cells. The latter could be attributed to the poor invasiveness of shNALCN PC-3 cells (see Figure 1F).

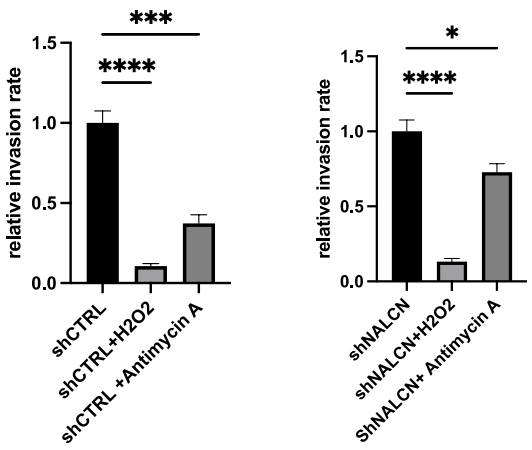


Figure. Effect of ROS on invasiveness of PC-3 cells at different NALCN bioavailability.

Critique R2.3: "*The data presentation can be improved and the manuscript could profit from a figure rearrangement. Following the main text and the red line of the study would thus be easier.* **"**

Response R2.3: We agree with the reviewer on this comment. To clarify the main message of the manuscript, we have modified virtually all the figures, both main and supplementary, and produced an appendix with two additional figures. In the revised version of our manuscript, virtually all the images are enlarged and the results of additional data analysis are presented where it was requested. That was done for the cost of omission of some data presented in the previous version of our manuscript.

Critique R2.4: *"The effects of NALCN manipulation on the therapeutic response of prostate cancer cells should be evaluated."*

Response R2.4: In accordance with your suggestion we have conducted the viability assays with MTS. We have assessed the effect of Docetaxel (chemotherapeutic drugs used in the treatment of patients with androgen-independent cancer) in the two groups of PC-3 cells: shCTRL and shNALCN.
We found that NALCN knockdown neither affected the viability of PC-3 cells nor augmented the effect of Docetaxel (at least within 4 days)

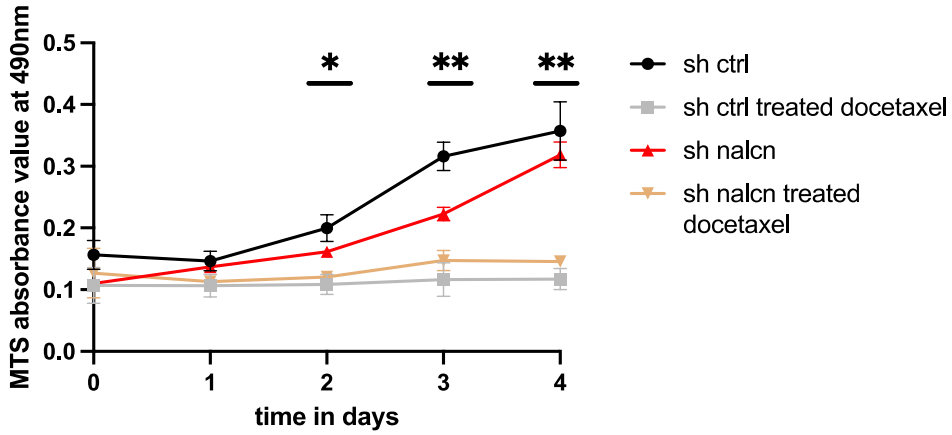


Figure. NALCN knockdown does not alter the effect of Docetaxel on PC-3 cells.

Minor comments:

Critique R2.5: **"***The abstract needs to be more compact and should more clearly describe the main findings as well as checked for typos. See for example: Mechanistically, NALCN associates functionally.... Or ..... Na+ leak channel, NALCN, at the hot spots..... (should be hot spot)*

Response R2.5: As requested by the reviewer abstract was modified.

Critique R2.6: "*The authors should discuss the role of ROS and SOC channels in cancer in more detail and in light of their current findings. How is it possible that mitochondrial ROS affect plasma membrane channels if the antioxidant systems in the cytosol are functional?"*

Response R2.6: We have directly demonstrated that pro-invasive factors (like FBS and EGF) induced Ca^{2+} oscillations not only in the cytoplasm but also in the ER and mitochondria (Figures 3 and 5). The ER origin of the Ca^{2+} events reported by Mag-Fluo-4 was confirmed by the expression of DsRed2-ER (Figure 3A). The mitochondrial origin of the Ca^{2+} events reported Rhod-2 was confirmed by double-staining with MitoTracker Green (Figure 5A). Furthermore, we have demonstrated that cytosolic [Ca^{2+}] oscillation reported by Fluo-4 were associated with oscillation of superoxide production by mitochondria reported by MitoSOX Red (Figure 5B).

To address the question of the Reviewer – *"How is it possible that mitochondrial ROS affect plasma membrane channels if the antioxidant systems in the cytosol are functional?"* – we have analyzed spatial re-organization of mitochondria following stimulation with 10% FBS of PC-3 cells expressing AcGFP-Mito. We found that mitochondria started to accumulate at the sites of invadopodia formation (see Figure below panel A and video Mito_PM24frs.avi) thus creating micro domains where mitochondrial ROS may affect the activity of the channels/transporters in the cell plasma membrane. Formation of NALCN-enriched "rosettes" at these sites was demonstrated in separate experiments (see Figure below panel B and video NALCN_PM24frs.avi).

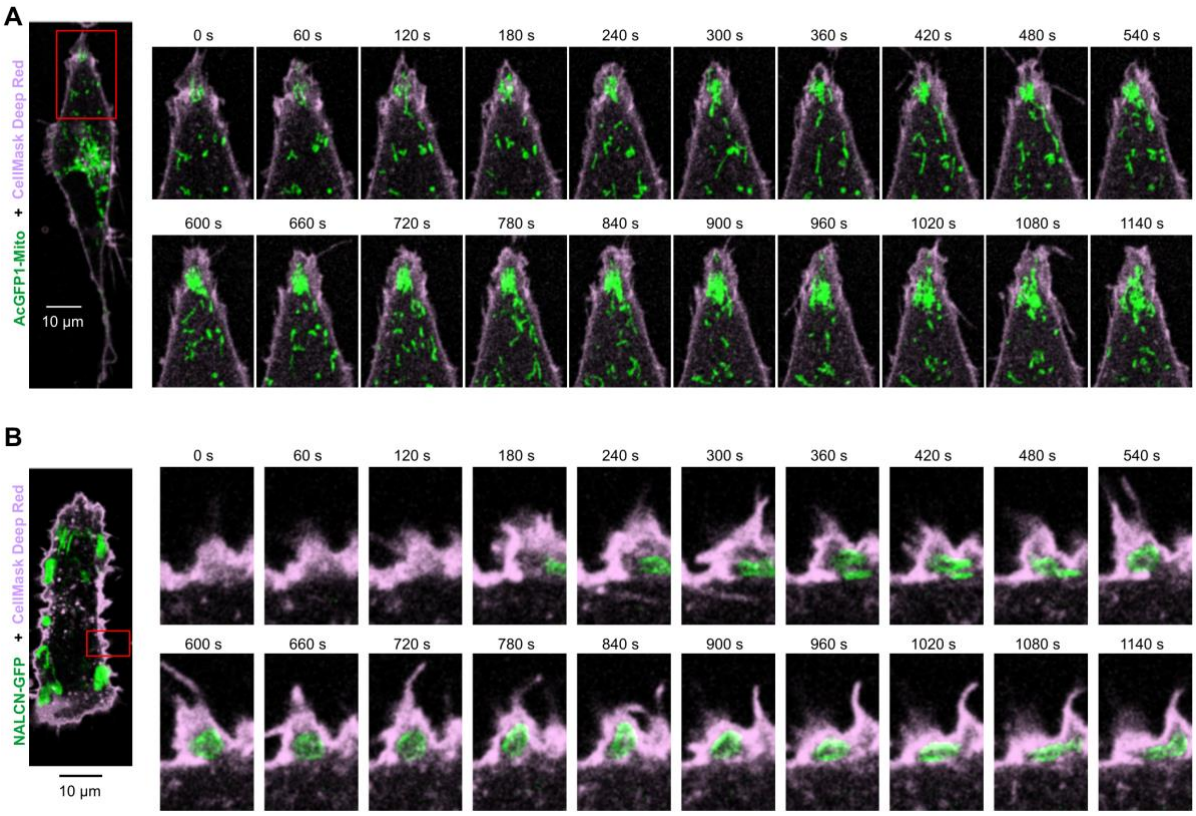


Figure. Accumulation of mitochondria and NALCN-enriched structures at the sites of invadopodia formation.
PC-3 cells expressing either AcGFP1-Mito (**A**) or NALCN-GFP (**B**) were stained with CellMask Deep Red. The x-y time series confocal imaging was performed at 0.1 Hz after stimulation of the cells with 10% FBS. The galleries (right) show every 6th image captured from the boxed regions (left). Note accumulation of mitochondria (**A**) and formation of NALCN-enriched "rosette" at the site of invadopodia formation. Please also see enclosed animations.

These data will be a part of separate article focused on regulation of plasmalemmal proteins by mitochondria that is currently in preparation.

Critique R2.7: *"The IHC images shown in Fig. 1 need to be quantified and statistically tested. Moreover, it has to be confirmed that in Fig 1c the differences in*

expression levels are due to the upregulation of NALCN in the cancer cells and not due to increased infiltration of other cell types such as immune cells."

Response R2.7: The reproducibility of IHC experiments was quantified and statistically tested, all of the results are presented in Figure 1C of the new version of the manuscript.

Critique R2.8: *How was the metastatic potential of the cells shown in Fig 1e determined? "*

Response R2.8: The metastatic potential was determined in accordance with current literature relating each cell line to the certain stage of cancer in the patients and according to *in vivo* preclinical models using these cell lines. However, since in our study we didn't use all cells lines for *in vivo* experiments we have removed this panel from the revised manuscript.

Critique R2.9: "*Migration and invasion assays (see Fig. 1f) should be performed in at least one additional cell line "*

Response R2.9: Thank you for your remark. These assays were done in another cell line: PC3-M. The results are shown below*:*

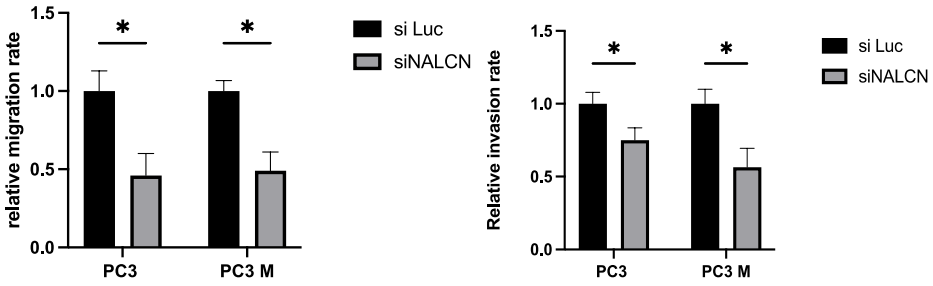


Figure. The effects of *NALCN* silencing on the migration and invasion rates of the cells from PC3-M cell line.

As can be seen, *NALCN* silencing has similar effect in both prostate cancer cell lines: the decrease of both the migration and invasion rates.

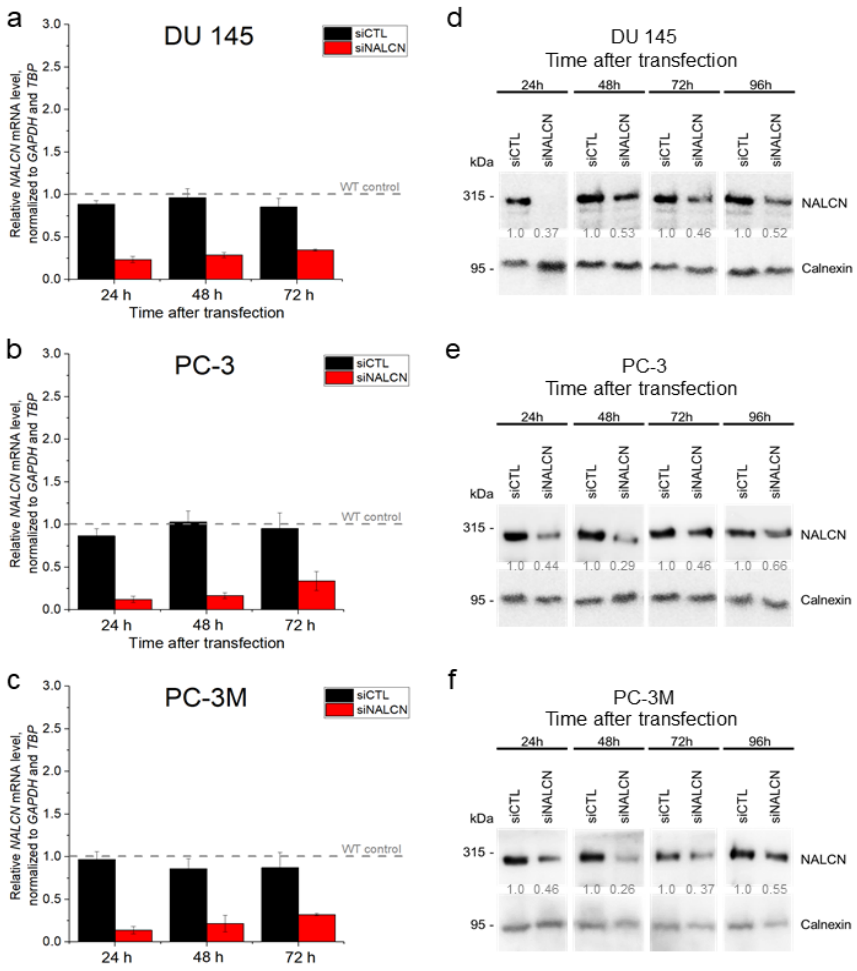
Due to the general reorganization of the figures, requested by both reviewers, these data are omitted in the revised version of the manuscript.

Critique R2.10: "*The supplementary figures should be reorganized and ordered as they are mentioned in the text".*

Response R2.10: Thank you. Done.

Critique R2.11: "*Please provide WB-based quantification of the shRNA efficacy."*

Response R2.11: Please find below the quantification by western blot of shNALCN in PC3 and other cell lines:



Critique R2.12: "*Images presented in Fig 2a should be quantified".*

Response R2.11: Done. Please see Figure 1F of the revised manuscript.

Critique R2.13: "*In Fig S2g only intracellular pH is measured. The text claims that both the extrusion and the intracellular pH was measured in S2g*

Response R2.13: The text was modified according to the Figure.

Critique R2.14: *"The authors describe the involvement of several proteins (Src, Tsk5, Cdc42etc) in the NALCN-controlled signaling axis. However, the rationale as to why exactly these proteins were selected is not clear. The paper would profit for more clear explanation in this regard."*

Response R2.14: These proteins (Src, Tks5, Cdc42) are considered as invadopodia markers and major players in invadopodia signaling inducing MMP secretion. We

included in the text of the revised manuscript the rationale for all these proteins. One of the best reviews providing most complete overwiev on the function of all the players in invadopodia formation and function is Murphy and Coutneidge “The ‘ins’ and ‘outs’ of podosomes and invadopodia: characteristics, formation and function” (Nature Reviews, Molecular Cell biology, 2011).

Critique R2.15: “ *Why was the Fura-2 signal normalized to 1? With ratiometric dyes this is not needed and wanted since the information about the resting calcium level is lost.* **“**

Response R2.15: Firstly, our study involves the number of imaging protocols utilizing ratiometric (e.g. Fura-2, SFBI) and non-ratiometric (e.g. Fluo-4, Rhod-2, Mag-Fluo-4, MitoSOX, FM1-43, mCherry, GFP) fluorophores. For unification of the presentation of the responses to stimuli over time we prefer presenting the traces of the responses on the scale of relative changes in the fluorescence intensities: $\Delta F/F_0$ for non-ratiometric probes and $\Delta R/R_0$ for ratiometric. This, on the one hand, allowed us statistical comparison of the responses by calculating mean signal temporal densities as masses of the signals ($\int(\Delta F / F_0)$ or $\int(\Delta R / R_0)$) per unite of time, while, on the other hand, makes comparison of the traces obtained with different fluorescent probes more feasible.

Secondly, Fura-2, with reported Kd to Ca^{2+} of 220-290 nM, may hardly be considered as precision tool for measuring resting $[Ca^{2+}]_c$, usually ranging around 10 nM or below.

Thirdly, our results suggest that long lasting oscillatory changes of $[Ca^{2+}]_c$ rather than changes in steady-state $[Ca^{2+}]_c$ are employed by the cells for encoding long-term effects.

Critique R2.16: *“All colocalization images should be quantified.”*

Response R2.16: As requested by the reviewer all colocalization images were quantified. Please see Figure 2A and Figure S1G of the revised manuscript.

Critique R2.17: *“Calcium channels have been reported to affect prostate cancer cell growth. However, the authors of this study do not observe such biological effects although calcium signaling is affected. The authors might want to comment on this. »*

Response R2.17: Indeed, a number of calcium channels (e.g. TRPV6, Orai1, Orai3) are known to affect prostate cancer cell growth (for review see Prevarskaya et al., Physiological Reviews 2018). In this study, we have demonstrated that, apart from providing the pathway for direct Ca^{2+} influx, these channels recruited to SOCE may also modulate Na^+ influx via NALCN and subsequent Ca^{2+} influx via the reverse-

mode plasmalemmal Na^+/Ca^{2+} exchanger (RM-NCX), thus giving rise to long-lasting $[Ca^{2+}]_c$ oscillations promoting invasion of cancer cells.

Dear Dr Prevarskaya,

Thank you for submitting your revised manuscript (EMBOJ-2022-112198R) to The EMBO Journal, as well as for your patience with our response at this time of the year. Your amended study was sent back to the two referees for their re-evaluation, and we have received comments from referee #2, which I enclose below. Please note that while reviewer #1 was at this time not able to give additional input, we have analyzed your response to his/her concerns editorially and found that the raised issues were satisfactorily addressed. As you will see, the other expert stated that the work has been substantially improved by the revisions and s/he is now in favour of publication, pending minor revision.

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

Please consider the remaining comments of the expert carefully and address them by reorganising data and the manuscript where appropriate, as well as amending the discussion of the findings.

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

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

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

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

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

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

****

Formatting changes required for the revised version of the manuscript:

>> Please limit the keywords to maximally five.

>> Adjust the title of the 'Competing Interests' section to 'Disclosure and Competing Interests Statement'.

>> Author Contributions: Remove the author contributions information from the manuscript text. Note that CRediT has replaced the traditional author contributions section as of now because it offers a systematic machine-readable author contributions format that allows for more effective research assessment. and use the free text boxes beneath each contributing author's name to add specific details on the author's contribution.

>> Figure callouts: the callout for Figure 1B should be placed after 1A.

>> Appendix: please add page numbers o the first page ToC.

>> Source Data: source data files need to be reorganized to one file/folder per figure and ZIPing for each main figure. For EV and/or appendix figures, ZIP together all source data.

>> Material and methods: please remove the numbering for the paragraphs. Add the current 'Study Approval' information to the 'in vivo xenografts' and 'Tissue biopsies' sections.

>> Remove the 'The paper explained' part from the manuscript.

>> Author checklist: please add information regarding human patient consent to the author checklist.

>> Consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.

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

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (2nd Jul 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #2:

The authors addressed most of the reviewers comments and the manuscript is improved. I have only minor suggestions mostly regarding the data presentation and interpretation.
1. I would suggest removing the term neuronal ion channels. In the abstract in particular this terminology might confuse non

expert readers. Neuronal channels in prostate cancer ..... Also the term neuron-like remodeling should be avoided.
2. The figures are revised but are still to full in my opinion. The manuscript would benefit a lot from relocating some of the data from the main figures to the supplementary information. Also adjusting the panel size to fit to a whole page should be avoided.

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Daniel Klimmeck, PhD
Senior Scientific Editor
The EMBO Journal
Heidelberg, Germany

May 5, 2023

Dear Dr. Klimmeck,

We are now re-submitting the revised version of our manuscript *"NALCN-mediated sodium influx regulates metastatic prostate cancer cell invasiveness"*.

Thank you for your kind letter from April 3 and for giving us an opportunity to do the modifications required for the final submission of the manuscript. We also thank both Reviewers for their time, attention, and helpful suggestions during revision.

We addressed all the comments and suggestions of the EMBO *Journal* and Referee 2. All formatting changes required for the revised version of the manuscript have been also done.

Referee 2 mentioned that "the manuscript would benefit a lot from relocating some of the data from the main figures to the supplementary information." However, for the previous revision we already removed a number of figures from main to the supplementary. As time for final revision of the manuscript is limited and as all the figures contain the images arranged in logical way and resizing of which could only

be done with keeping X-Y proportion, we prefer to keep the current structure and sequence of the figures.

As required for EMBO *Journal*, we also prepared ‘Synopsis’ containing the new figure and the sentences that summarize the article.

We hope that in light of all these modifications you can now find our work worthy of publication in *EMBO Journal*.

Kind regards

Natalia Prevarskaya and co-authors

Reply to reviewer comments.

Formatting changes required for the revised version of the manuscript:

>> Please limit the keywords to maximally five.
Done, as requested

>> Adjust the title of the 'Competing Interests' section to 'Disclosure and Competing Interests Statement'.
Done, as requested

>> Author Contributions: Remove the author contributions information from the manuscript text. Note that CRediT has replaced the traditional author contributions section as of now because it offers a systematic machine-readable author contributions format that allows for more effective research assessment. and use the free text boxes beneath each contributing author's name to add specific details on the author's contribution.
Done, as requested

>> Figure callouts: the callout for Figure 1B should be placed after 1A.
The callout for Figure 1B is now placed after 1A. Corresponding changes are introduced to the Figure 1 and Figure 1 Legend: panes B and C are swapped.

>> Appendix: please add page numbers o the first page ToC.
Done, as requested

>> Source Data: source data files need to be reorganized to one file/folder per figure and ZIPing for each main figure. For EV and/or appendix figures, ZIP together all source data.
Done, as requested

>> Material and methods: please remove the numbering for the paragraphs. Add the current 'Study Approval' information to the 'in vivo xenografts' and 'Tissue biopsies' sections.
Done, as requested

>> Remove the 'The paper explained' part from the manuscript.
Done, as requested

>> Author checklist: please add information regarding human patient consent to the author checklist.
Done, as requested

>> Consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.
Done, as requested, we considered comments, answered them, and left changes in track mode.

Referee #2:

The authors addressed most of the reviewers comments and the manuscript is improved. I have only minor suggestions mostly regarding the data presentation and interpretation.
1. I would suggest removing the term neuronal ion channels. In the abstract in particular this terminology might confuse non expert readers. Neuronal channels in prostate cancer ..... Also the term neuron-like remodeling should be avoided.
Done, as requested

2. The figures are revised but are still to full in my opinion. The manuscript would benefit a lot from relocating some of the data from the main figures to the supplementary information. Also adjusting the panel size to fit to a whole page should be avoided.
Many thanks for your suggestion. However, as time for final revision of the manuscript is limited and as all the figures contain the images arranged in logical way and resizing of which could only be done with keeping X-Y proportion, we prefer to keep the current structure and sequence of the figures.

2nd Revision - Editorial Decision **10th May 2023**

Dear Dr. Prevarskaya,

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

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

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. I would thus like to ask for your consent on keeping the additional referee figures included in this file.

Also, in case you might NOT want the transparent process file published at all, you will also need to inform us via email immediately. More information is available here:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

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

On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author(s) of the study. Please see the following link for representative examples and their integration into the article web page:
https://www.embopress.org/video_synopses
https://www.embopress.org/doi/full/10.15252/embj.2019103932

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

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

Thank you for this contribution to The EMBO Journal and congratulations on a successful publication!

Please consider us again in the future for your most exciting work.

Kind regards,

Daniel Klimmeck

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EMBO Press Author Checklist

Corresponding Author Name: Prevarskaya Natalia
Journal Submitted to: THE EMBO JOURNAL
Manuscript Number: EMBOJ-2022-112198R

USEFUL LINKS FOR COMPLETING THIS FORM

The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:

  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Not Applicable	

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknolegments and/or Author section

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	In each figures
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	In each figures and Materials and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	In each figures
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	In each figures

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Yes	Materials and Methods and data sources Figure 1B
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	