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Redox signals at the ER-mitochondria interface control melanoma progression

Xin Zhang, Christine S. Gibhardt, Thorsten Will, Hedwig Stanisz, Christina Körbel, Miso Mitkovski, Ioana Stejerean, Sabrina Cappello, David Pacheu-Grau, Jan Dudek, Nasser Tahbaz, Lucas Mina, Thomas Simmen, Matthias W. Laschke, Michael D. Menger, Michael P. Schön, Volkhard Helms, Barbara A. Niemeyer, Peter Rehling, Adina Vultur, Ivan Bogeski

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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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 21st Nov 2018

Thank you for the submission of your manuscript (EMBOJ-2018-100871) to The EMBO Journal. Your manuscript has been sent to three referees, and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and novelty of your work, although they also express a number of major issues that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In more detail, referee #2 states that your claims on direct causalities between TMX activity and NFAT1 are not sufficiently supported by the current data, which in his/her view undermines the impact of your findings (ref#2, pt.1). In addition, this referee asks you to consolidate the findings by additional Ca2+ activation and depletion / SOCE assays (ref#2, pts.2,4). Referee #1 states issues regarding the unresolved BRAF-dependence of the phenotype and requests clarification. In line, referee #3 points to concerns regarding the generality and physiological in vivo relevance of the results. In addition, the referees point to issues related to experimental design and lack of critical controls that would need to be conclusively addressed to achieve the level of robustness 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. I need to stress though that we do need string support from the referees on a revised version of the study in order to move on to publication of the work.

REFEREE REPORTS:

Referee #1:

In the present manuscript, Zhang et al., have studied the role of TMX1-3 oxidoreductase/NFAT1 axis in melanoma stage and metastatic potential. They have demonstrated how disrupting ER-mitochondria communication by TMX downregulation affected cellular redox processes and NFAT1 translocation. I think the topic of this article, is indeed interesting and important. There are data from several other labs, which have established the role of ROS in melanoma oncogenesis. This report seems to go further in identifying additional actors in this process. The paper is written clearly and of adequate extent. In results, the experiments are clearly presented. Statistical analysis was properly performed. The discussion is sound and comprehensive. The design of this study has been well-realized including in vitro experiments with multiple human melanoma cell lines, xenografted-mice, samples from human melanoma patients (and healthy skin melanocyte) and meta-analyses (Protein Atlas, Gene Ontology Consortium, differential gene expression datasets).

Major Concerns:
(1) A significant proportion of advanced melanomas (about 40-50%) harbors a mutation in the BRAF gene (eq: V600E). Therapies with BRAF and MEK inhibitors are associated with significant long-term treatment benefit in patients with BRAF V600-mutated melanoma. Molecular testing for BRAF mutations is now a priority in determining the course of therapy. As written by the authors, " it was reported that targeting NFAT signaling enhanced melanoma cell death in oncogenic BRAF cells. The two major cell lines used in this paper are WM3734 (BRAFV600E) and Mel Juso (BRAFwt). But the authors have not addressed the question of TMX1/TMX3/NFAT axis individually in the both context BRAFV600E or BRAFWT. The role of BRAF in this context needs to be determined (eq: using BRAF RNA interference) and authors should also repeat meta-analysis in both groups (patients BRAFV600E vs BRAFwt) to answer to this important question.

Minor concerns:
(1) Authors have written that « TMX1 downregulation induces elevated ROS production in mitochondria by inducing Ca2+ overload ». But this link has not been clearly demonstrated. Authors could complete these experiments using Ca2+ chelator (eq: BAPTA, AM ester) to determine the importance of calcium in this context. In the manuscript, authors stipulate that the increase of mitochondrial ROS production has been associated to Ca2+ overload and alteration in mitochondrial morphology and intracellular positioning. But OXPHOS enhancement (as seen Fig S6H) could be sufficient to explain ROS production because elevated mitochondrial Ca++ levels allosterically stimulate the activity of 3 TCA cycle enzymes. This issue needs to be clarified.

(2) Statistic analyses are missing in Fig S1A and S2B

Referee #2:

The manuscript by Zhang et al identifies a novel TMX-NFAT axis, which is upregulated in melanoma and negatively correlates with patients survival. TMX resides at the ER-mitochondria interface and controls ROS production. TMX knockdown enhances ROS production and inhibits NFAT nuclear import via a ROS-dependent mechanism. TMX and NFAT are required for melanoma cell proliferation. Finally, TMX knockdown in a mouse xenograft model causes a transient decrease in tumor growth.

Overall, the manuscript explores a novel and interesting role for redox signaling generated at MAMs in melanoma progression. However, some issues must be clarified before publication.

1) While TMX is upregulated in all melanoma specimens and most of melanoma cell lines, NFAT expression is more heterogeneous. Thus, in some melanoma that require TMX for growth and progression, NFAT may be dispensable. To clarify whether TMX still sustains cell growth and migration in tumors that do not express NFAT, and to check whether ROS are involved, the authors should perform the following experiments: in WM1366 (that lack NFAT1 expression, see fig 1B-C) cells upon TMX silencing they should a. measure H2O2 levels; b. measure cell growth and

migration; c. monitor tumor growth in mouse xenografts.
2) In Figure 2, it is not clear why the authors chose insulin to trigger NFAT nuclear import, since in all other experiments NFAT nuclear import was induced by thapsigargin treatment. In addition, in parallel to NFAT nuclear import, show cytosolic Ca2+ measurements in insulin-treated cells. Similarly, show cytosolic Ca2+ measurements in tg-treated cells.
4) In Figure 3, the authors show that TMX kd does not affect SOCE. However, differently from Figure 2, in Figure 3 they induce SOCE by Ca2+ addition following ER Ca2+ depletion. SOCE in TMX kd cells should be measured in the same conditions of Figs1C-H, i.e., by thapsigargin addition in Ca2+-containing medium. In addition, basal cytosolic [Ca2+] should be quantitatively reported.
5) The authors show that TMX controls NFAT nuclear import by regulating ROS levels independently of SOCE. However, whether tg treatment affects ROS levels in melanoma cell lines is unknown. Please measure cytosolic H2O2 levels in tg-treated cells. Related to figures 4C-D, please measure NFAT nuclear import upon tg treatment in NAC-treated control cells. Finally, ROS measurements in TMX1 kd +/- NAC/catalase/DTT should be performed.
6) According to data of figs 5G-J and to published literature, TMX1 kd should affect ER-mitochondria contacts, which in turn might affect mitochondrial Ca2+ uptake, respiration and mitochondrial ROS production. However, quantification of mitochondria-ER contacts in control vs TMX1 kd melanoma cells is lacking. This experiment is mandatory in order to understand the mitochondria-related parameters.
7) Fig S6H suggests that TMX stable knockdown clones (TMX1 kds1 and kds2) increases OCR. Since stable clones behave differently from transient kd cells (e.g. figures S6F-G), OCR measurements should be performed also in transient TMX silenced cells (TMX kd cells).
8) Are mitochondrial H2O2 levels reduced in TMX1 kd cells by ionomycin or rotenone treatment? Does ionomycin or rotenone treatment revert the effects of TMX1 kd on NFAT nuclear import and on melanoma cell growth and migration?
9) The authors suggest that mitochondria positioning in proximity to the PM could account for the increased mitochondrial Ca2+ uptake in TMX1 kd cells. Is this process ROS dependent? Does antioxidant treatment in TMX1 kd cells restore mitochondria morphology, mitochondrial Ca2+ uptake and OCR?
10) Do TMX1 kd mitochondria express the same levels of the MCU and of the NCLX? Why mitochondrial Ca2+ uptake was measured upon addition of 0.25 mM Ca2+ (Fig. 5D), while cytosolic Ca2+ increase was measured upon addition of 1 mM Ca2+ (Figure 3A)? Please use the same experimental conditions to measure basal, as well as Ca2+ induced, cytosolic and mitochondria [Ca2+].
11) Figure 5J: why HeLa cells have been used for TEM? A similar experiment should be performed in WM3734 cells.
12) Are the effects on proliferation and migration of TMX1 kd reverted by antioxidant treatment?
13) In all figure legends, statistical analysis and tests used to assess significance must be specified.

Minor:
1) Figure S1D: please show NFAT1 expression in these human melanoma samples.
2) Figure 1C: please show quantification of protein expression.
3) Figure 1D panel 5: please indicate TMX1 positive melanocytes.
4) Figure S3: please measure calcineurin activity in WM3734 cells in the presence of NAC and catalase respectively.
5) It is not clear which time point is represented in figure S6F. Is this the endpoint of the xenograft experiment? P-AKT/AKT, as well as TMX1 protein levels should be reported both at 19 days post-grafting and at 45 day post-grafting, in order to understand why differences in tumor growth observed after 19 days are lost after 45 days post-grafting. In the same figure, it is not clear what C4 sample is. There are two samples named C4, one on the left blot and one on the right one. The one on the left is obscure. What is A3 (control)? Are those TMX1 kds cells before xenograft?

Referee #3:

In this work Zhang and colleagues propose a critical role for TMX1 and TMX3 in promoting growth of melanoma cell lines. They also claims that these genes can be considered as prognostic markers

of melanoma proliferation and invasion. They also proposed a quite complex redox and Ca2+-dependent control of NFAT1 function by TMX. Last, but not least, the authors provide a bioinformatic analyses of Cancer Genome Atlas data to support a role for NFAT1 in tumor associated gene and TMX to affect disease outcome.
Overall, the manuscript is filled of data that are not necessarily informative and/or appropriate to provide conclusions. Rather in the current form these data are quite confusing.
The conclusions are not supported from the data in many sections. A better way would be to analyze the phenotype and, provide a hypothesis and then, tested it.
The correlation of TMX function in melanoma (progression) is based on IHC analyses and xenograft assays. Rather, more specific in vivo experiments should be provided (e.g. KO model for TMX1/3, PDTX models) to support a conclusive and direct role of TMX in melanoma and the molecular link with NFAT1. I would definitely rewrite the paper being more cautiousness.

Major suggestions/questions:
Major suggestions/questions:

1. Since the authors showed that only WM3734 has high TMX1 and NFAT1 expression from qRT-PCR and WB data (they performed Western for 7 cell lines from 10), but not for the other cell lines, it is not possible to claim that the data obtained here are related to melanoma.
2. Authors did use Mel Juso cells without showing expression of protein NFAT1and TMX1 by WB. The use of SK Mel5 would have been a better control. Also, Authors used in 1 experiment 1205Lu cells, which are not the best model from their data (fig. 6D).
3. Fig 3. These experiments on general ROS production are pointless if not associated to an ER-localised or mitochondrial localized ROS detection. Targeted version of Hyper or RoGFP2 probes should be use here. The useful data are shown in Figure 5, instead.
4. Fig 4. Tapsigargin induced ROS production in these cells ? What are the levels of ROS among the different melanoma cell lines used in these work ? Is there a correlation between the levels of TMX1 and redox state in all cell lines tested ?
5. Figure 5. The connection between ROS and NFAT translocation is weak. A molecular mechanism should be proposed and/or tested. What' s the molecular relationship between NOX4 and TMX ? Is this just epiphenomena or there is a molecular link ?
6. Figure 6: Mild phenotypes are achieved both in vitro and in vivo. In vitro migration assays cannot recapitulate tumor invasion phenotype. Also, the in vivo exp are just xenograft and are not measuring invasion.
7. I don't see the important and specific role of TMX3 here. Authors mentioned the separate role of TMX3 as a prognostic marker in the table S2, but that's it. Usually, they described the role of TMX3 in accordance with TMX1 in some experiments. Is it redundant compared to TMX1 ?.
8. Not sure that 2 samples for IHC were statistically significant: it might better to have 3 samples for each group.
9. I am not certain that is correct to use data from experiments with HeLa cells (fig. 5I-J) to show an increase in mitochondrial exposure to the PM. These figures are representative, but it is not melanoma.
10. I suggest to place the section «Fura-based Ca2+-imaging» under the section «Fluorescence microscopy» in Materials and Methods.
11. I suggest to write the section «Statistical analysis» in Materials and Methods.
12. It is better to mark molecular weight on all Western blot figures.

Minor suggestions/questions:

13. Page 8 section «NFAT nuclear translocation is impaired in TMX-silenced melanoma cells»: it is better to explain and provide a rationale why these melanoma cell lines were selected in the text. Also, Fig 1E should be moved to Supplementary information and enlarged. It is difficult to identify cellular information at this resolution.
14. Fig S1A/B lacks statistical analyses.
15. Pag. 9. I would remove TMX3 from the title of the results section.
16. Page 10, 2nd paragraph, link to the Fig4H-I in the text: I think authors could mention that these data obtained from MelJuso cells,
17. Page 12, paragraph 1st, link to the fig. 5I-J: they should mention that is HeLa cell line,
18. Page 14, paragraph 1st: authors did not show that TMX1 knockdown is stable, might be that is

why tumor growth is stopped to grow for a while,
19. Page 23, section «Cell culture and reagents»: might be it is better to mention that all cell lines were checked for their cellular identity with appropriate markers (see journal policy).
20. Page 23, section «Cell culture and reagents», 12th line: «and» is repeated twice,
21. Page 24, section «Fura-based imaging»: more details about the microscope, camera, objective and how Ca2+ was evaluated,
22. Page 25, section «Fluorescence microscopy»: more details of the microscope, conditions of imaging: temperature, CO2...
23. Page 25, section «Ca2+ and hydrogen peroxide measurements»: how FRET was measured, equation,
24. Page 26, 2nd paragraph: how the protein was extracted, more details of phosphatase activity measurement,
25. Page 27, section RT-qPCR: how was mRNA isolated and thermo-cycling conditions,
26. Page 27, section «Immunoblotting»: dilution of antibodies is not written,
27. Page 27, section «Determination of mitochondrial value and surface»: which cells? how were determined volume and surface, formula?
28. Page 29, section «Immunohistochemistry»: how images were acquired?
29. Page 29, section «In vivo studies»: how many mice were in each group, gender and age, at which size tumors were fixed?
30. Page 38, Fig 1C: I think it is better to do western for all 10 melanoma cells with quantification of density, especially for TMX1,
31. Page 46, Fig 7A: GOI could be deciphered,
32. Page 52, Fig S5B: GAPDH bands are saturated too much, there is no space below the bands in blots of Mel Juso and SK Mel5; it not clear which band to analyse on BiP blot for
Mel Juso cells (there are 3 bands),
33. Page 54, Fig. S6 F-G: pAkt and Akt bands are not similar in the band width and the horizon, and spots, they could be 2 different membranes, but there is 1 control.

1st Revision - authors' response 22nd Mar 2019

We thank the reviewers and the editorial board for the overall positive evaluation of our study, for the constructive comments and for giving us the opportunity to revise our manuscript. As seen in this point-to-point reply, we performed a substantial number of new experiments, which are included in the revised version of the manuscript. We believe that the new data strengthen our experimental findings and conclusions to improve the overall quality of this study.

Point-by-point reply:

For convenience, we include the revised figures also within this point-to-point reply. These figures are titled Figure XX or Figure EVXX as found in the revised manuscript.

Results that are not included in the manuscript but are useful for addressing the reviewers' concerns and suggestions are also included. These figures are titled Figure_Rev_XX.

Editor:

Thank you for the submission of your manuscript (EMBOJ-2018-100871) to The EMBO Journal. Your manuscript has been sent to three referees, and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and novelty of your work, although they also express a number of major issues that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In more detail,

referee #2 states that your claims on direct causalities between TMX activity and NFAT1 are not sufficiently supported by the current data, which in his/her view undermines the impact of your findings (ref#2, pt.1).

Our results show that NFAT1 controls mitochondrial bioenergetics and promote NFAT1 as an important regulator of melanoma aggressive behavior, similar to PGC1α, MITF and JARID1B (please see manuscript discussion for details). In addition, we demonstrate that disturbing the ER-mitochondrial communication by silencing TMX1 or TMX3 leads to elevated mitochondrial ROS. Accordingly, NFAT1 positive cells have more energized mitochondria, which can generate higher levels of ROS, in particular under stress conditions (i.e. disturbances in the ER-mitochondrial communication) and thus inhibit NFAT1 via the oxidation of calcineurin. According to our data, this mitochondria-ROS-NFAT1 feedback loop might be an Achilles' heel of aggressive, NFAT1-positive melanoma subgroups.

Based on the reviewers' and editor's comments, and in order to dissect the TMX-NFAT1 connection in more detail, we analyzed the role of TMX1 and TMX3 in NFAT1 translocation, ROS production, cell growth, migration and invasion in the NFAT1-negative WM1366 cells. In addition, we performed *in vivo* xenografts with TMX1 silenced WM1366 cells.

In addition, this referee asks you to consolidate the findings by additional Ca2+ activation and depletion / SOCE assays (ref#2, pts.2,4).

We apologize for not including all controls. These experiments have now been performed. For details, please see our response to reviewer 2's comments.

Referee #1 states issues regarding the unresolved BRAF-dependence of the phenotype and requests clarification.

As suggested by the reviewer, we performed new bioinformatic analyses from the TCGA-derived patient database. Moreover, we made a qPCR-based correlative analysis between the BRAF genotype and TMX1, TMX3 and NFAT1 mRNA expression in a panel of melanoma cell lines used in this study. Furthermore, using siRNA against BRAF, we addressed the role of BRAF in NFAT1 nuclear translocation. For details, please see our response to reviewer 1's comments.

In line, referee #3 points to concerns regarding the generality and physiological in vivo relevance of the results.

To expand our understanding on the generality of the TMX-ROS-NFAT1 axis in melanoma, we evaluated the role of TMX silencing on NFAT1 translocation, ROS production, proliferation, migration and invasion in additional melanoma cell lines, including the NFAT1-negative WM1366. With the additional TCGA database analysis and the WM1366 xenograft model, we evaluate the *in vivo* relevance of our findings in more detail.

In addition, the referees point to issues related to experimental design and lack of critical controls that would need to be conclusively addressed to achieve the level of robustness needed for The EMBO Journal.
We apologize for not including all controls. This issue is addressed in the revised manuscript.

Referee #1:
In the present manuscript, Zhang et al., have studied the role of TMX1-3 oxidoreductase/NFAT1 axis in melanoma stage and metastatic potential. They have demonstrated how disrupting ER-mitochondria communication by TMX downregulation affected cellular redox processes and NFAT1 translocation. I think the topic of this article, is indeed interesting and important. There are data from several other labs, which have established the role of ROS in melanoma oncogenesis. This report seems to go further in identifying additional actors in this process. The paper is written clearly and of adequate extent. In results, the experiments are clearly presented. Statistical analysis was properly performed. The discussion is sound and comprehensive. The design of this study has been well-realized including in vitro experiments with multiple human melanoma cell lines, xenografted-mice, samples from human melanoma patients (and healthy skin melanocyte) and meta-analyses (Protein Atlas, Gene Ontology Consortium, differential gene expression datasets).
We thank the reviewer for the positive evaluation of our study.

Major Concerns:
(1) A significant proportion of advanced melanomas (about 40-50%) harbors a mutation in the BRAF gene (eq: V600E). Therapies with BRAF and MEK inhibitors are associated with significant long-term treatment benefit in patients with BRAF V600-mutated melanoma. Molecular testing for BRAF mutations is now a priority in determining the course of therapy. As written by the authors, " it was reported that targeting NFAT signaling enhanced melanoma cell death in oncogenic BRAF cells. The two major cell lines used in this paper are WM3734 (BRAFV600E) and Mel Juso (BRAFwt). But the authors have not addressed the question of TMX1/TMX3/NFAT axis individually in the both context BRAFV600E or BRAFWT. The role of BRAF in this context needs to be determined (eq: using BRAF RNA interference) and authors should also repeat meta-analysis in both groups (patients BRAFV600E vs BRAFwt) to answer to this important question.
We thank the reviewer for this very helpful suggestion.
Indeed, we have chosen the two cell lines (WM3734 and Mel Juso) because of their different BRAF status. The fact that we observe similar effects in both lines following TMX silencing suggested that the TMX-ROS-NFAT1 signaling axis is functionally relevant in both BRAF V600E and BRAF WT melanoma cells.
As suggested, to explore the role of BRAF status on the TMX-ROS-NFAT1 axis in more detail, we performed bioinformatic analyses on the TCGA melanoma patient

database. As seen in Figure 7C-D and Appendix Table S4, out of 97 patients, 49 were BRAF WT and 48 BRAF V600E. We first evaluated the effect of BRAF V600E on NFAT1, TMX1 and TMX3 expression levels and found that NFAT1 transcripts are only significantly elevated in patients carrying the BRAF V600E mutation (two-sided Wilcoxon Rank Sum test p=0.0007) while TMX1 and TMX3 levels were not significantly affected (Fig. 7B). To examine the BRAF-NFAT1 interplay in more detail, we divided the BRAF WT and BRAF V600E groups in NFAT1-high and NFAT1-low subgroups (Appendix Table S4). This approach indicated that in the BRAF V600E group, more patients had high NFAT1 (n_high=26 vs n_low=22) compared with the WT group (n_high=15 vs n_low=34, Fisher's exact test, p=0.024). As in Fig. 7B neither TMX1 nor TMX3 showed systematic changes of their expression levels when BRAF WT and BRAF V600E cohorts were compared.

To examine the clinical relevance of the NFAT1-BRAF relationship, we evaluated the survival probability of the patients categorized as NFAT1-high versus the patients categorized as NFAT1-low in the BRAF WT population. We found that the survival probability is significantly reduced (log-rank test p=0.0022) in the patients with high NFAT1, when compared with the patients with low NFAT1 (Fig. 7C). Furthermore, we performed the same analysis in the BRAF V600E patient group and found that in this case, NFAT1 expression is not relevant for the survival probability (Fig. 7D).

In addition, the qPCR-based correlational analysis of the BRAF status and NFAT1, TMX1 and TMX3 expression in the panel of cell lines used in this study (Figure EV5Q-S) showed similar relationship (increased NFAT1 in the BRAF V600E cells and unchanged TMX1 and TMX3) as in the human patient samples.

Moreover, downregulation of BRAF using siRNA did not prevent the TMX1 silencing-induced inhibition of NFAT1 nuclear translocation (Figure EV5O-P).

Our new data suggest that BRAF V600E melanomas express higher levels of NFAT1, while TMX1 and TMX3 levels are BRAF-“insensitive”. However, BRAF WT melanomas also express NFAT1 and the BRAF WT and NFAT1-high combination causes severe decrease in survival expectancy.

Overall our results display the complexity and interconnected signaling of NFAT1 and BRAF in melanoma. Nevertheless, within this signaling network, our data show clearly that the TMX-ROS-NFAT1 signaling axis is functionally relevant in both BRAF WT and BRAF V600E melanomas.

	NFAT1		**TMX1**		**TMX3**	
	low	high	low	high	low	high
WT	34	15	38	11	30	19
V600E	22	26	28	20	25	23
Fisher's exact test	p=0.024		n.s		n.s	

Appendix Table S4: The role of BRAF on NFAT1, TMX1 and TMX3 expression in melanoma patients

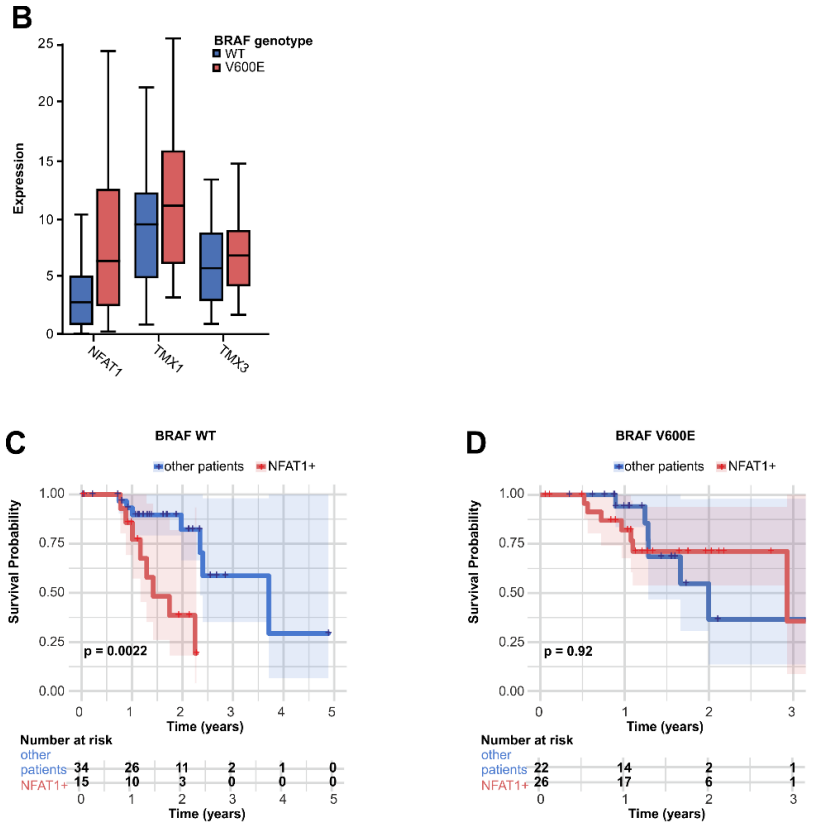


Figure 7B, C and D: The TMX-ROS-NFAT1 signaling axis in BRAF WT and BRAF V600E melanoma patients.

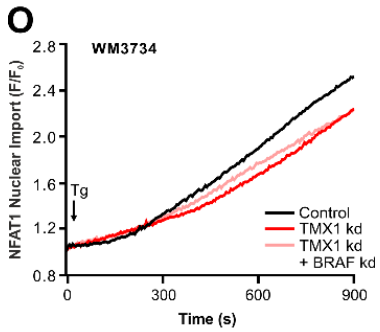


Figure EV5O: BRAF does not affect the TMX1 silencing-induced inhibition of NFAT1 nuclear translocation

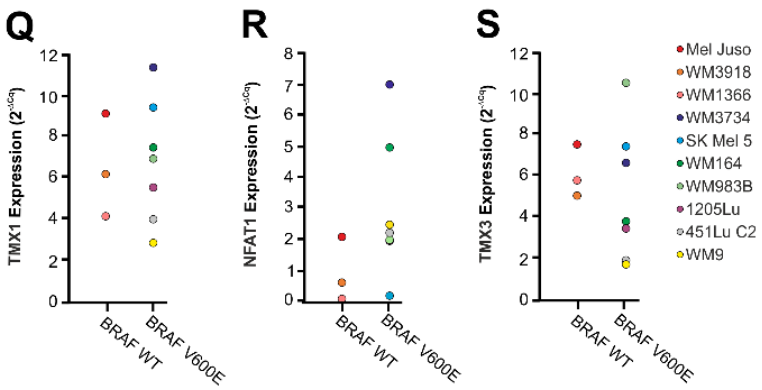


Figure EV5 Q, R, S: The BRAF status does not affect TMX1 and TMX3 expression in melanoma cell lines

Minor concerns:

(1) Authors have written that « TMX1 downregulation induces elevated ROS production in mitochondria by inducing Ca2+ overload ». But this link has not been clearly demonstrated. Authors could complete these experiments using Ca2+ chelator (eq: BAPTA, AM ester) to determine the importance of calcium in this context. In the manuscript, authors stipulate that the increase of mitochondrial ROS production has been associated to Ca2+ overload and alteration in mitochondrial morphology and intracellular positioning. But OXPHOS enhancement (as seen Fig S6H) could be sufficient to explain ROS production because elevated mitochondrial Ca++ levels allosterically stimulate the activity of 3 TCA cycle enzymes. This issue need to be clarified.

We agree with the reviewer that calcium overload may play an important role. Accordingly, we evaluated the effects of BAPTA-AM on H_2O_2 production following TMX1 downregulation as suggested.

Our data shown in Fig. EV4K suggest that BAPTA-AM treatment only partially reduced the TMX silencing-induced elevation in mitochondrial H_2O_2, thus suggesting that calcium signals are important, but not the only regulators of TMX silencing-induced ROS production.

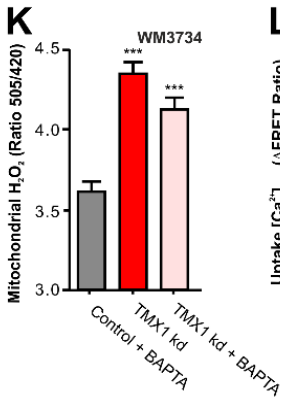


Figure EV4K: BAPTA-AM partially reduces the TMX1 silencing-induced ROS production

(2) Statistic analyses are missing in Fig S1A and S2B
Statistics for Fig. S1A and S1B are now provided in Fig. EV1A and B. We also performed additional experiments for higher accuracy. These new data are also incorporated.

Referee #2:
The manuscript by Zhang et al identifies a novel TMX-NFAT axis, which is upregulated in melanoma and negatively correlates with patients survival. TMX resides at the ER-mitochondria interface and controls ROS production. TMX knockdown enhances ROS production and inhibits NFAT nuclear import via a ROS-dependent mechanism. TMX and NFAT are required for melanoma cell proliferation. Finally, TMX knockdown in a mouse xenograft model causes a transient decrease in tumor growth.
Overall, the manuscript explores a novel and interesting role for redox signaling generated at MAMs in melanoma progression. However, some issues must be clarified before publication.
We thank the reviewer for the positive evaluation of our work.

1) While TMX is upregulated in all melanoma specimens and most of melanoma cell lines, NFAT expression is more heterogeneous. Thus, in some melanoma that require TMX for growth and progression, NFAT may be dispensable. To clarify whether TMX still sustains cell growth and migration in tumors that do not express NFAT, and to check whether ROS are involved, the authors should perform the following experiments: in WM1366 (that lack NFAT1 expression, see fig 1B-C) cells upon TMX silencing they should a. measure H2O2 levels; b. measure cell growth and migration; c. monitor tumor growth in mouse xenografts.
We agree with these suggestions and have performed the experiments suggested by the reviewer under points a., b. and c.

a) Measurements of cellular H_2O_2 and pH in TMX1 silenced WM1366 cells are shown in Fig. EV3F and G. As depicted, TMX1 silencing caused elevated H_2O_2 also in the NFAT1-negative WM1366.

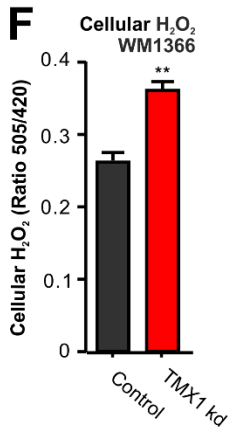


Figure EV3F: TMX1 knockdown causes H_2O_2 production in the NFAT1 negative WM1366 melanoma cells

b) As seen in Fig. EV5B and C, TMX1 or TMX3 silencing caused a reduction in WM1366 cell growth. However, when compared with WM3734 and Mel Juso cells, this effect is less pronounced (10 % vs 19 % and 21 % for TMX1 and 7 % vs 29 % and 39 % for TMX3, respectively). WM1366 cells originate from a tumor in vertical growth phase and are less aggressive than the WM3734 and the Mel Juso cells; thus, direct comparison is not trivial. In addition, we also measured migration in TMX1 and TMX3 silenced cells. Figure EV5C, shows no significant differences in transwell migration between the silenced and the control cells.

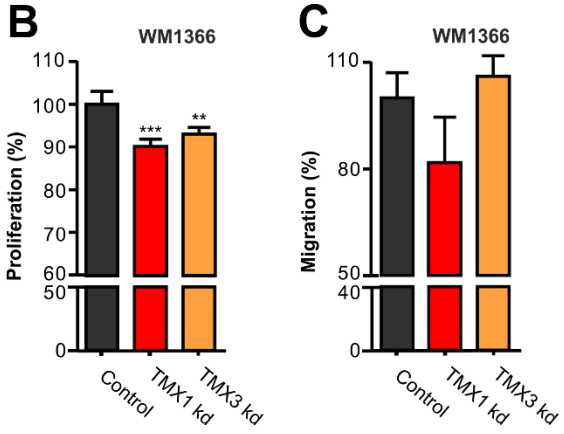
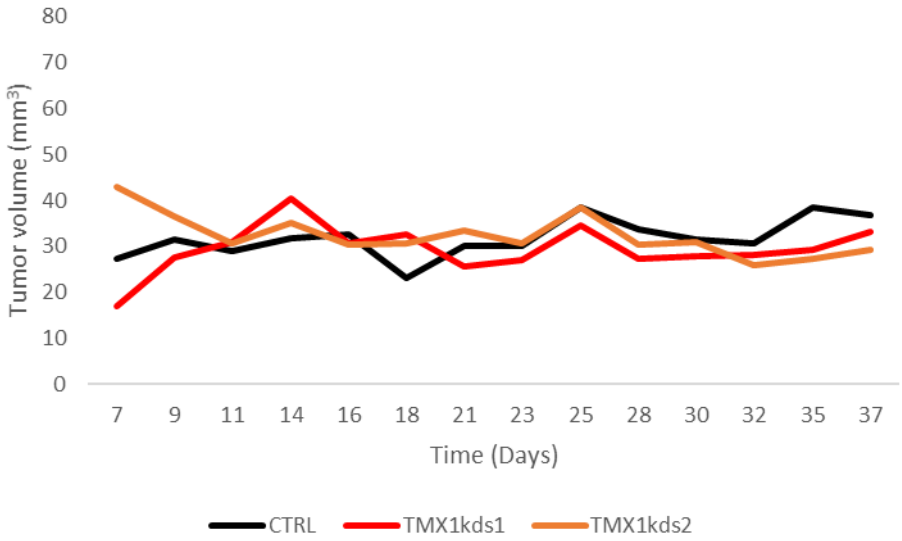


Figure EV5B and C: TMX1 or TMX3 silencing causes a slight inhibition in proliferation and has no effect on transwell migration in the NFAT1-negative WM1366 melanoma cells

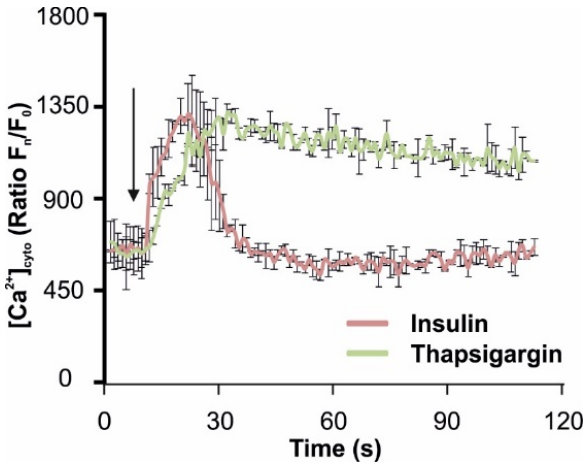
c) As suggested, we have performed melanoma cell WM1366 xenografts same as for the WM3734 cell line (see Figure_Rev 1). As shown, even after 37 days, the tumors failed to substantially grow. We believe that this is due to the origin of the WM1366 (less aggressive, vertical tumor growth phase) and to their negative NFAT1 status, which indicates the low aggressiveness of these cells. Accordingly, the findings from the WM1366 xenografts indirectly support our conclusions regarding the role of NFAT1 in melanoma aggressive behavior.



Figure_Rev_1: WM1366 xenografts in immunodeficient NSG mice

2) In Figure 2, it is not clear why the authors chose insulin to trigger NFAT nuclear import, since in all other experiments NFAT nuclear import was induced by thapsigargin treatment. In addition, in parallel to NFAT nuclear import, show cytosolic Ca2+ measurements in insulin-treated cells. Similarly, show cytosolic Ca2+ measurements in tg-treated cells.

We thank the reviewer for pointing this out. Insulin was chosen to demonstrate that physiological stimulation of store operated Ca^{2+} entry (SOCE) also induces NFAT1 nuclear translocation. We have tested the effects of insulin on SOCE in WM3734 cells before performing the NFAT1 import experiments but did not include the data in the original manuscript. Please see the requested measurements in Figure_Rev_2. These results are included in a doctoral thesis that we now cite in the manuscript.



Figure_Rev_2: Insulin induces Ca^{2+} entry in WM3734 cells

Measurements of store operated Ca^{2+} entry in WM3734 cells. Cells were exposed to insulin (1.75 µg/mL) or Tg (1 µM) as indicated. Data are presented as mean ± SEM.

4) In Figure 3, the authors show that TMX kd does not affect SOCE. However, differently from Figure 2, in Figure 3 they induce SOCE by Ca^{2+} addition following ER Ca^{2+} depletion. SOCE in TMX kd cells should be measured in the same conditions of Figs1C-H, i.e., by thapsigargin addition in Ca^{2+}-containing medium. In addition, basal cytosolic [Ca2+] should be quantitatively reported.

We agree that showing these controls is important. The requested experiments have now been performed using two calcium indicators (Fura-2AM and the genetically encoded cytosolic Ca^{2+} FRET sensor D_3cpV). The data shown in Fig. 3A-C and Fig. EV3A-C demonstrate no overt differences in SOCE in TMX1 or TMX3 knockdown cells vs control.

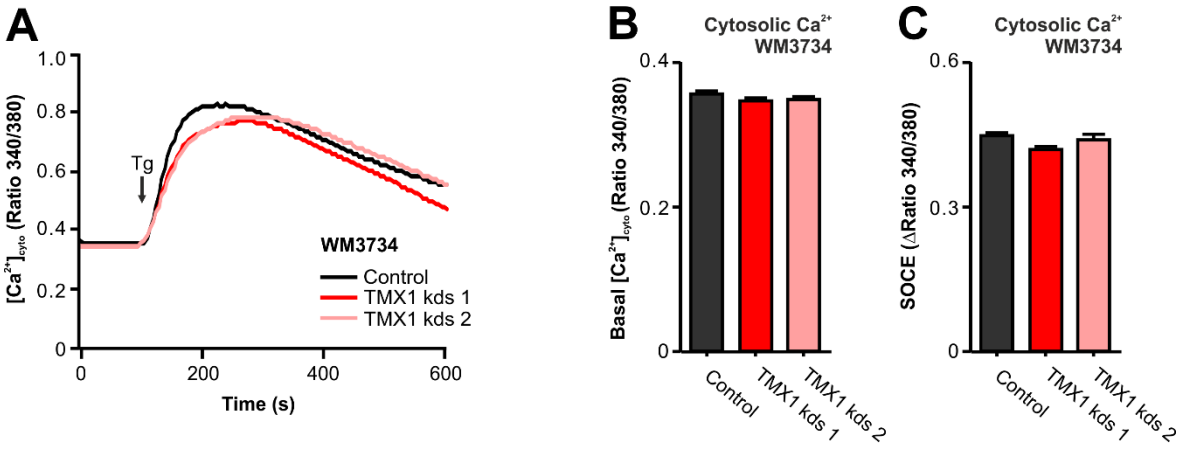


Figure 3A-C: Fura-2AM-based calcium measurements demonstrate no overt differences in SOCE following TMX1 downregulation

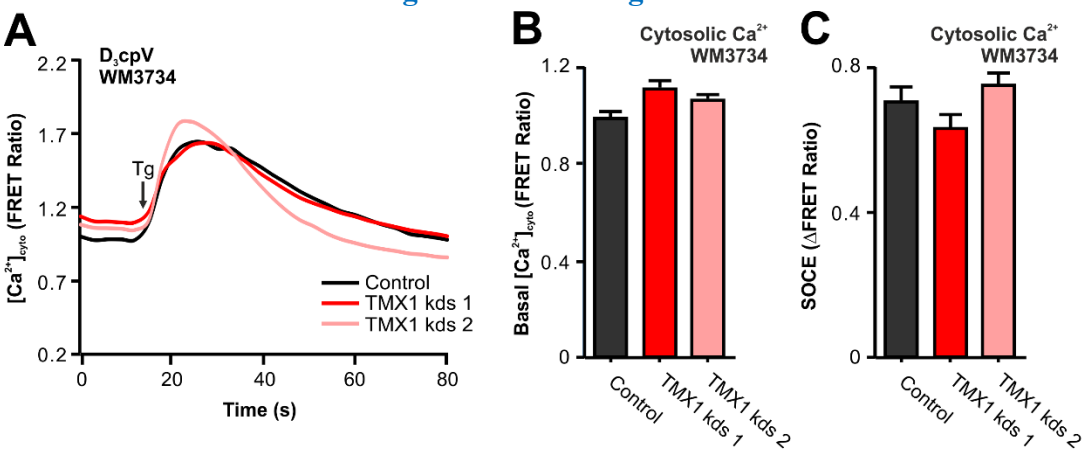


Figure EV3A-C: D_3cp $V_{cytosolic}$-based calcium measurements demonstrate no overt differences in SOCE following TMX1 downregulation

5) The authors show that TMX controls NFAT nuclear import by regulating ROS levels independently of SOCE. However, whether tg treatment affects ROS levels in melanoma cell lines is unknown. Please measure cytosolic H2O2 levels in tg-treated cells. Related to figures 4C-D, please measure NFAT nuclear import upon

tg treatment in NAC-treated control cells. Finally, ROS measurements in TMX1 kd +/- NAC/catalase/DTT should be performed.

We agree with the reviewer. We have now measured the effect of Tg on cellular H_2O_2 in WM3734 cells. The data depicted in Fig. EV3H indicate that acute Tg-treatment is not affecting the global ROS levels in WM3734 cells.

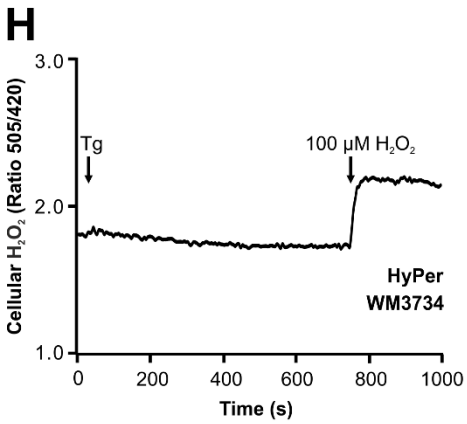


Figure EV3H: Thapsigargin has no overt influence on cellular H_2O_2

The NFAT1 nuclear import upon Tg treatment was also measured in NAC-treated control cells. The data shown in Fig. 4D indicated no significant effect of NAC on the Tg-induced NFAT1 translocation.

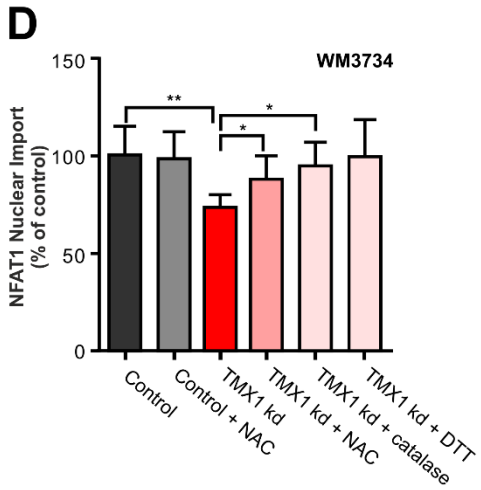


Figure 4D: Antioxidants do not affect Tg-induced NFAT1 nuclear translocation *(compare black to gray)*

The effects of NAC, catalase and DTT on cellular ROS have been evaluated and are now presented in Fig. EV3M. As expected, all agents significantly reduced the HyPer signals. It has to be mentioned that DTT will directly reduce the redox sensitive cysteine in HyPer and is thus not providing additional information on TMX silencing-induced ROS production.

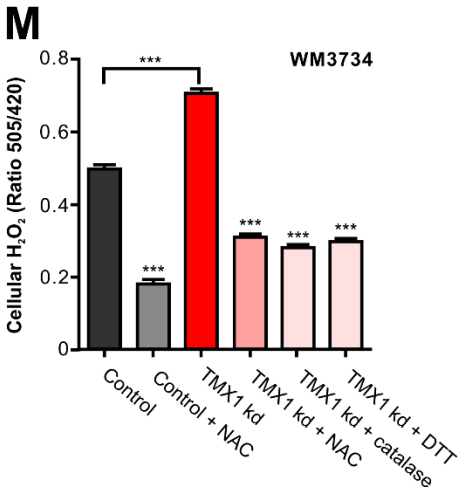


Figure EV3M: NAC, catalase and DTT reduce HyPer ratio

6) According to data of figs 5G-J and to published literature, TMX1 kd should affect ER-mitochondria contacts, which in turn might affect mitochondrial Ca2+ uptake, respiration and mitochondrial ROS production. However, quantification of mitochondria-ER contacts in control vs TMX1 kd melanoma cells is lacking. This experiment is mandatory in order to understand the mitochondria-related parameters.

We agree. We performed these experiments using 3D confocal microscopy and electron microscopy. The new data shown in Fig. 5I and J demonstrate higher presence of mitochondria near the plasma membrane in TMX1 silenced vs control melanoma cells. Electron microscopy of melanoma cells confirmed these findings and demonstrated decreased MAM lengths and increased ER-mitochondria distances (see Fig. 5K-N). For details, please also see Methods, Figure 9 and Results section in the MS.

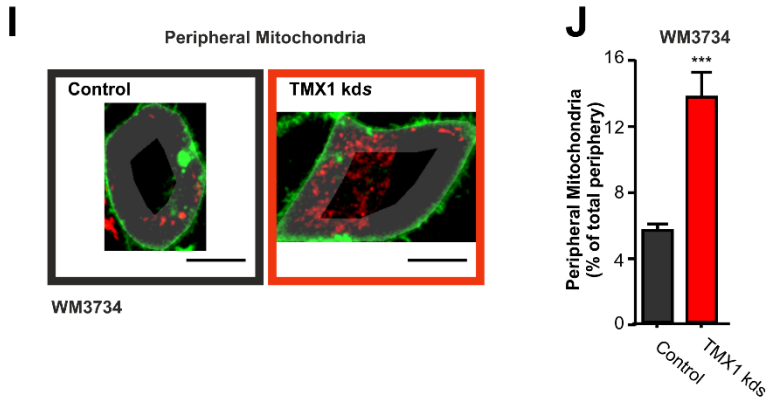


Figure 5I and J: TMX1 silencing elevates mitochondrial content in the vicinity of the plasma membrane in melanoma cells (confocal fluorescent imaging).

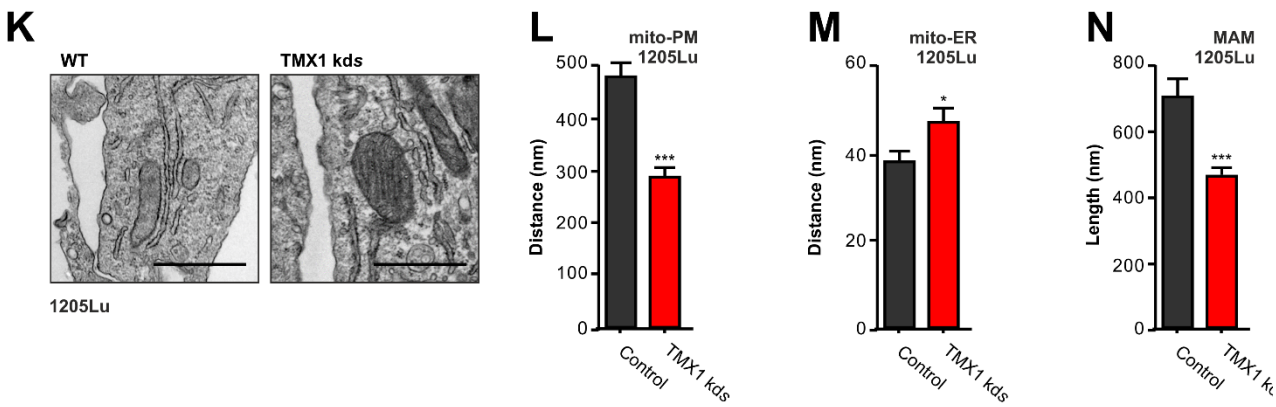
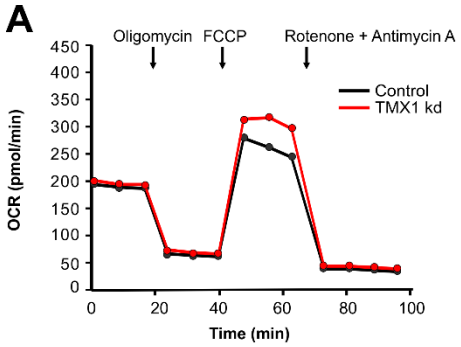


Figure 5K-N: TMX1 silencing decreases mitochondria-plasma membrane distances, causes shorter MAM domains and elevates ER-mitochondria distances in melanoma cells (electron microscopy).

7) Fig S6H suggests that TMX stable knockdown clones (TMX1 kds1 and kds2) increases OCR. Since stable clones behave differently from transient kd cells (e.g. figures S6F-G), OCR measurements should be performed also in transient TMX silenced cells (TMX kd cells).

The data shown in Figure_Rev_3 indicate that maximal respiration is also increased in WM3734 cells transiently silenced for TMX1. This effect was, however, not as prominent as in the stably transfected cells (see Fig. EV5G), thus suggesting that a fraction of the elevated respiration might be due to adaptive remodeling of mitochondrial respiration machinery.



Figure_Rev_3: Transient silencing of TMX1 causes elevated mitochondrial respiration

8) Are mitochondrial H2O2 levels reduced in TMX1 kd cells by ionomycin or rotenone treatment? Does ionomycin or rotenone treatment revert the effects of TMX1 kd on NFAT nuclear import and on melanoma cell growth and migration?

Ionomycin is an ionophore which is commonly used to raise the intracellular calcium concentration, while rotenone inhibits complex I in the mitochondrial electron transfer chain (ETC). Both compounds are toxic and cause cell death due to calcium overload or oxidative stress, respectively (J Biol Chem. 2002 Jul 26;277(30):27217-26).

Treating the cells with ionomycin will elevate intracellular calcium concentration and thus cause NFAT1 translocation (Proc Natl Acad Sci U S A. 1995 Nov 21;92(24):11205-9). Hence, examining its effect on the Tg-induced NFAT1 nuclear translocation in cells silenced for TMX1/3 will most likely not be possible.
Rotenone prevents electron flow from complex I to Coenzyme Q10 causing electrons to escape the mitochondrial ETC and to reduce the molecular oxygen to superoxide that readily dismutates into hydrogen peroxide. Rotenone will thereby elevate mitochondrial and cellular ROS and cause apoptosis independently of TMX1 or NFAT1 (J Biol Chem. 2003 Mar 7;278(10):8516-25).
Summarized, we thank the reviewer for these suggestions and agree that deeper understanding of the mitochondrial calcium-ROS interplay was needed. However, due to our concerns about the interpretation of the data and bearing in mind the new results within the revised manuscript, which also address these issues, we decided to focus on the other experiments suggested by the reviewer.

9) The authors suggest that mitochondria positioning in proximity to the PM could account for the increased mitochondrial Ca2+ uptake in TMX1 kd cells. Is this process ROS dependent? Does antioxidant treatment in TMX1 kd cells restore mitochondria morphology, mitochondrial Ca2+ uptake and OCR?
We agree with the reviewer that redox regulation of the MCU complex might affect mitochondrial calcium uptake as we and others have already reported (Cell Metab. 2015 Oct 6;22(4):721-33 and Mol Cell. 2017 Mar 16;65(6):1014-1028.e7). Moreover, mitochondrial dynamics can be regulated by redox signaling (Cell Metab. 2015 Aug 4;22(2):207-18), see also our manuscript discussion.
Antioxidants are very potent agents that are able to affect a number of signaling mechanisms within cells. Accordingly, their usage and interpretation of the obtained data needs to be executed with care. We have performed the suggested experiments and observed that antioxidant treatment does not significantly affect mitochondrial Ca^{2+} uptake (Fig. EV4L). In addition, antioxidants did not affect the elevated mitochondrial respiration in the TMX1 knockdown cells (see Figure_Rev_4 below).

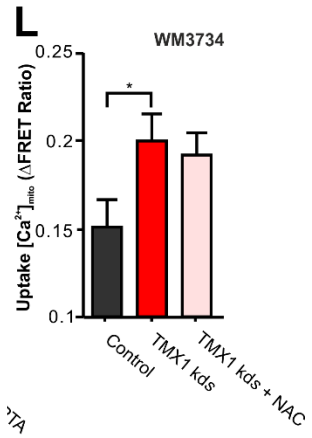
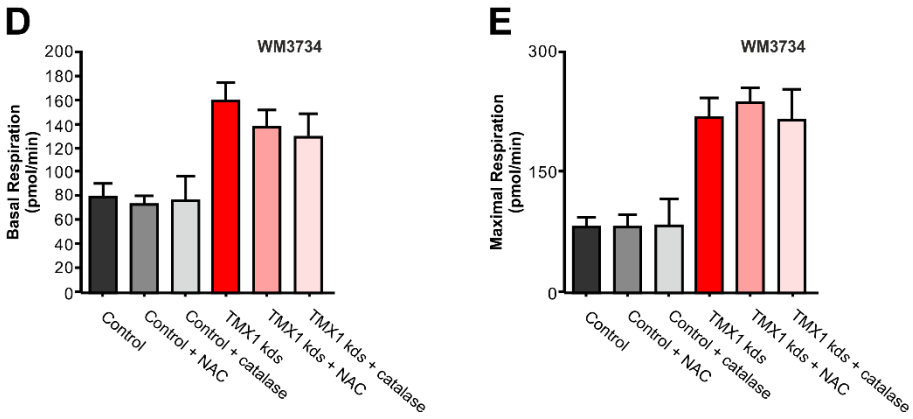


Figure EV4L: NAC has no significant effect on TMX1 silencing-induced mitochondrial Ca^{2+} increase



Figure_Rev_4: Antioxidants do not affect the TMX1 silencing-induced increase in mitochondrial respiration

10) Do TMX1 kd mitochondria express the same levels of the MCU and of the NCLX? Why mitochondrial Ca2+ uptake was measured upon addition of 0.25 mM Ca2+ (Fig. 5D), while cytosolic Ca2+ increase was measured upon addition of 1 mM Ca2+ (Figure 3A)? Please use the same experimental conditions to measure basal, as well as Ca2+ induced, cytosolic and mitochondria [Ca2+].

We thank the reviewer for suggesting these important controls. We have examined the expression levels of MCUa, MCUb and NCLX upon TMX downregulation using qPCR and WB analyses. The obtained results are presented in Fig. EV4M and N and demonstrate no overt i.e. consistent differences in MCU and NCLX expression following TMX silencing in WM3734 and WM1366 cells.

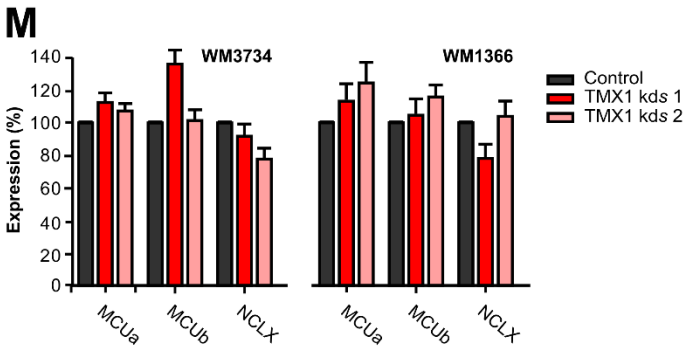


Figure EV4M: qPCR-based evaluation of MCUa, MCUb and NCLX transcripts shows no significant alterations following TMX1 silencing in melanoma cells

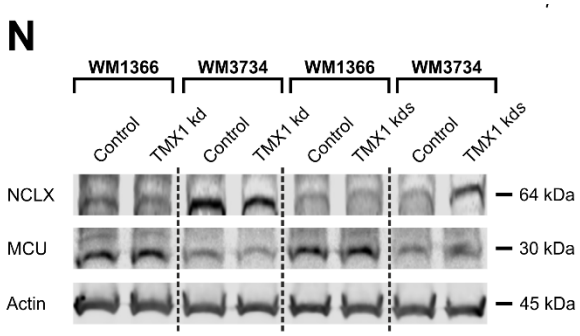


Figure EV4N: WB-based evaluation of MCU and NCLX abundance shows no significant alterations following TMX1silencing in melanoma cells

As suggested by the reviewer, we performed additional calcium measurements to achieve uniform experimental conditions. For all calcium-related experiments within this study, we used an external bath with 0.25 mM Ca^{2+} and for the most important experiments we, in addition, used an external bath solution with 1 mM Ca^{2+}.

Note: point 11 was missing.

12) Are the effects on proliferation and migration of TMX1 kd reverted by antioxidant treatment?
We performed the suggested experiments and observed reversal of the TMX silencing-induced inhibition of cell growth and migration by antioxidant treatment (Fig. 6C and Fig. 6E). However, as mentioned above, antioxidants may affect alternative signaling pathways, which are not influenced by TMX silencing-induced ROS. This may partially affect the interpretation of our experimental data. For example, two recent studies showed that antioxidants promote melanoma metastatic spread (Sci Transl Med. 2015 Oct 7;7(308):308re8 and Nature. 2015 Nov 12;527(7577):186-91.). This note is also mentioned in the revised manuscript.

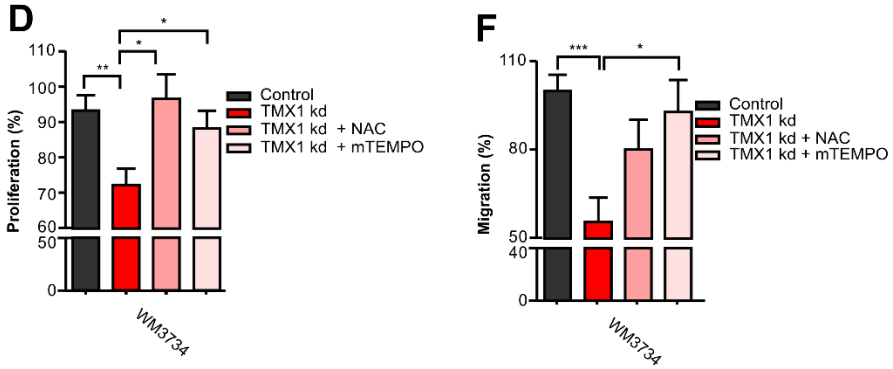


Figure 6D and F: Antioxidants reverse the inhibitory effects of TMX1 silencing on proliferation and migration of melanoma cells

13) In all figure legends, statistical analysis and tests used to assess significance must be specified.
We apologize for this. This has now been corrected. To avoid lengthy figure legends, some of the information is provided in the materials and methods section.

Minor:
1) Figure S1D: please show NFAT1 expression in these human melanoma samples.
The conclusions and comparisons drawn in the manuscript are supported by the data in Fig. 1 (with multiple samples). Figure EV1D just confirms the correlation between TMX1 and aggressive disease. Obtaining patient samples is not trivial and we unfortunately, do not have the possibility to stain samples from the same patients for NFAT1 (some are very rare melanomas). If Fig. EV1D is confusing to readers (and given that it just confirms the findings in Fig. 1), we could remove this dataset. Nevertheless, we feel that it would be a pity not to present this information to the interested reader.

2) Figure 1C: please show quantification of protein expression.
The quantification of protein expression in Figure 1C is now included and the western blot was updated to include all melanoma cell lines as shown for the qPCR in figure 1A and B.

3) Figure 1D panel 5: please indicate TMX1 positive melanocytes.
Figure 1D shows that NFAT1 is present only in the tumor lesion while TMX1 is found in the lesion, but also in healthy cells including melanocytes. Staining the same section with a melanocyte marker was, unfortunately not possible, but we do show the melanocytes in parallel sections of the same sample (panels 1 and 2).

4) Figure S3: please measure calcineurin activity in WM3734 cells in the presence of NAC and catalase respectively.
The results of the new calcineurin activity measurements are depicted in Fig. 4J and K. The new data show that antioxidant treatment reverses the TMX1 silencing-induced calcineurin inhibition similar as for NFAT1 nuclear translocation.

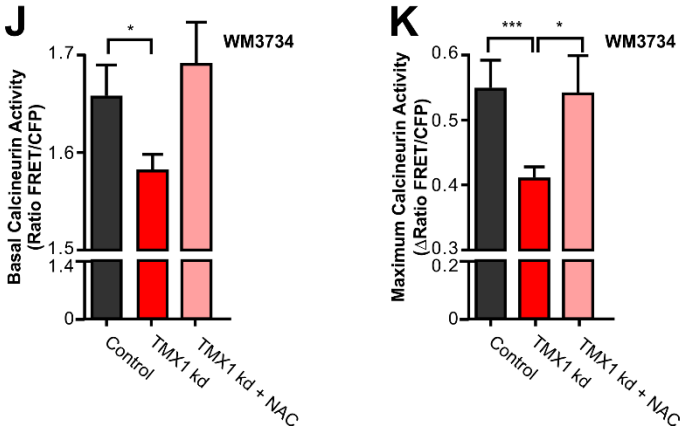


Figure 4J and K: The TMX1 silencing-induced inhibition of calcineurin activity is reversed by antioxidant treatment

5) It is not clear which time point is represented in figure S6F. Is this the endpoint of the xenograft experiment?
Yes, this is the endpoint; this is now indicated more clearly in the text, in the figure and in the figure legend.

P-AKT/AKT, as well as TMX1 protein levels should be reported both at 19 days post-grafting and at 45 day post-grafting, in order to understand why differences in tumor growth observed after 19 days are lost after 45 days post-grafting.
In vivo tumor protein analyses require tumor extraction and animal sacrifice according to our animal protocols. We understand the reviewer's interest in what happens on day 19; however, this requires 2 additional animal experiments (one for day 19 and one repeat of what we already showed for day 45, to be run in parallel to allow comparison with day 19). Unfortunately, this approach may not easily answer the question posed, as upregulation and compensation of multiple pathways can occur over many days and not just upon seeing a shift in growth. The purpose of the AKT analyses on day 45 were in order to highlight this point, i.e. compensatory pathways are engaged to offset TMX knockdown *in vivo* and this indicates the biological relevance of TMX in melanoma. TMX knockdown was confirmed in the cell lines prior to *in vivo* injection and was again confirmed on day 45 upon tumor extraction (Figure EV5D), indicating stable knockdown over time.

In the same figure, it is not clear what C4 sample is. There are two samples named C4, one on the left blot and one on the right one. The one on the left is obscure. What is A3 (control)? Are those TMX1 kds cells before xenograft?
We apologize for the confusion and have now improved the presentation and labeling of our data. The labeled "A" "B" "C" samples are all *in vivo* tumor lysates. "A" referring to knockdown controls where TMX levels remain high. "B" and "C" have TMX1 knocked down (same target, different shRNA). In the first submission, "C4" was shown in duplicate to show reproducibility/signal stability. We agree that this can confuse rather than help readers and we now simplified the figure accordingly. We also improved the figure labels.

Referee #3:

In this work Zhang and colleagues propose a critical role for TMX1 and TMX3 in promoting growth of melanoma cell lines. They also claim that these genes can be considered as prognostic markers of melanoma proliferation and invasion. They also proposed a quite complex redox and Ca2+-dependent control of NFAT1 function by TMX. Last, but not least, the authors provide a bioinformatic analyses of Cancer Genome Atlas data to support a role for NFAT1 in tumor associated gene and TMX to affect disease outcome.

Overall, the manuscript is filled of data that are not necessarily informative and/or appropriate to provide conclusions. Rather in the current form these data are quite confusing.

The conclusions are not supported from the data in many sections. A better way would be to analyze the phenotype and, provide a hypothesis and then, tested it.

We thank the reviewer for this suggestion. Indeed, we have considered presenting our data as this reviewer suggests before submitting the manuscript, but decided to use the present form instead. We still believe that the current presentation is more appropriate in describing our findings in an optimal way. If the reviewer and the editorial board agree, we would favor keeping the current format.

The correlation of TMX function in melanoma (progression) is based on IHC analyses and xenograft assays. Rather, more specific in vivo experiments should be provided (e.g. KO model for TMX1/3, PDTX models) to support a conclusive and direct role of TMX in melanoma and the molecular link with NFAT1. I would definitely rewrite the paper being more cautiousness.

We believe that this comment might be connected with the interpretation of the term “progression”. Generating a melanocyte-specific TMX KO mouse model would take a lot of time and would still not address the role of TMX in human melanoma, since mouse skin features distinct biology from human skin (J Cell Commun Signal. 2016 Sep; 10(3): 191–196). Moreover, a TMX KO model would provide information regarding the role of TMX in tumor initiation, but in human melanomas, TMX changes may not be among the original genetic “hits”, rather other mutations are likely to drive initial tumorigenic events. The focus of our paper is not to suggest that TMX is an initiator of melanoma, but that it contributes to a more aggressive disease.

Regarding the use of PDTX models: Given that no known drugs exist that selectively affect TMX function; we believe that performing PDTX experiments for this study using the genetic manipulation of TMX will also require *in vitro* cell line expansion, thus defeating the purpose of using the PDTX in the first place. Due to the extensive characterization of the patient samples used and the selection of TMX knockdown cells, *in vitro* work is necessary; for example, PDTX would require a full TMX/NFAT/ROS/Ca^{2+} characterization. We note here that our mouse studies are secondary to and only support the more important information provided by the patient samples (IHC and bioinformatics data).

Major suggestions/questions:

1. Since the authors showed that only WM3734 has high TMX1 and NFAT1 expression from qRT-PCR and WB data (they performed Western for 7 cell lines from 10), but not for the other cell lines, it is not possible to claim that the data obtained here are related to melanoma.

We are thankful for this suggestion. We now provide WB analysis for all requested melanoma cell lines (Fig. 1C). In addition, we quantified NFAT1 translocation and observed similar effects of TMX1 silencing in three additional melanoma cell lines (Figure EV2J, L and N). These new results further support the important role of the TMX-ROS-NFAT1 axis in melanoma.

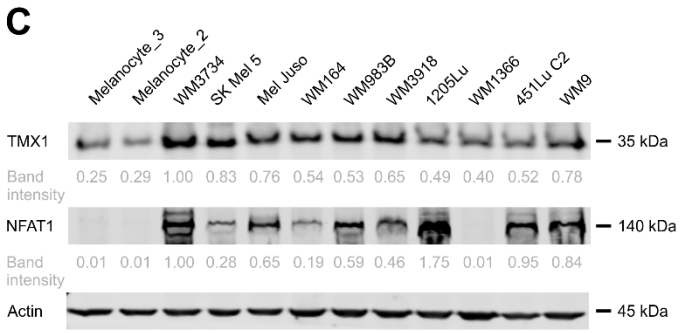


Figure 1C: TMX1 and NFAT1 abundance in a panel of genetically distinct melanoma cells (see Table S1 for details on cell line genetic background)

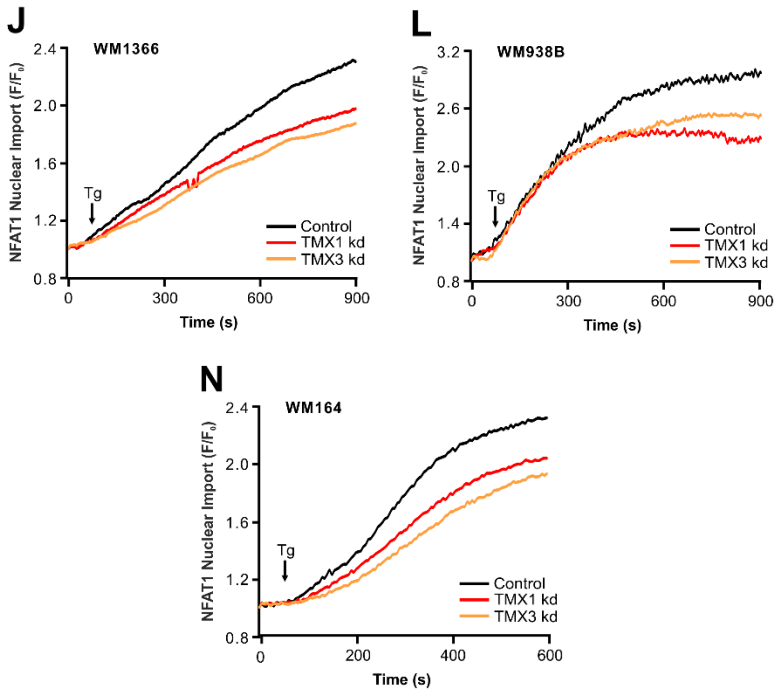


Figure EV2J, L and N: TMX1 silencing causes NFAT1 inhibition in WM1366, WM938B and WM164 melanoma cells

2. Authors did use Mel Juso cells without showing expression of protein NFAT1and TMX1 by WB. The use of SK Mel5 would have been a better control.

Also, Authors used in 1 experiment 1205Lu cells, which are not the best model from their data (fig. 6D).

We apologize for not providing western blot analysis for the Mel Juso cell line. This is now corrected (please see Fig. 1C above). We have also used the 1205Lu cell line for additional experiments and together with the NFAT1-negative WM1366, we now have four main cell lines for our study.

As seen in Table S1, Mel Juso cells are BRAF WT while SK Mel 5 are BRAF V600E. Because WM3734 cells are also BRAF V600E, we selected Mel Juso in order to conduct observations that are not BRAF-specific, but instead are more likely to apply to multiple melanoma cell subgroups. 1205Lu is one of the best characterized melanoma xenograft models published; it is aggressive and metastatic to the lung. This line was chosen to allow comparisons with other publications.

Please also see our reply to Reviewer 1 and the Results section for detailed explanation regarding the melanoma cell lines chosen for this study.

3. Fig 3. These experiments on general ROS production are pointless if not associated to an ER-localised or mitochondrial localized ROS detection. Targeted version of Hyper or RoGFP2 probes should be use here. The useful data are shown in Figure 5, instead.

NFAT1 in its "inactive" phosphorylated state resides in the cytosol. Accordingly, it was important to examine the cytosolic ROS levels following TMX downregulation. To detect the origin of these ROS, we proceeded to measure mitochondrial ROS levels (Figure 5A-C in the MS, as commented by the reviewer). We also measured ROS in the ER, but decided not to show these data because all available ROS sensors are (at least partially) oxidized in the ER lumen due to the highly oxidizing luminal redox potential. These results are now presented in Figure EV4A-C and demonstrate ER H_2O_2 levels that are slightly but significantly higher in the TMX1 knockdown cells (Fig. EV4C). However, these differences might be greater if a sensor that remains fully reduced in the ER-lumen would have been available. Figure S5A depicts that the ER-HyPer probe is oxidized in the ER lumen independently of TMX1 and can be thus reduced by DTT. That the probe is not fully oxidized is shown in Fig. EV4B (external H_2O_2 can still increase the ER-HyPer ratio). We included these data in the manuscript, together with the discussion regarding the technical limitations of measuring ROS within the ER.

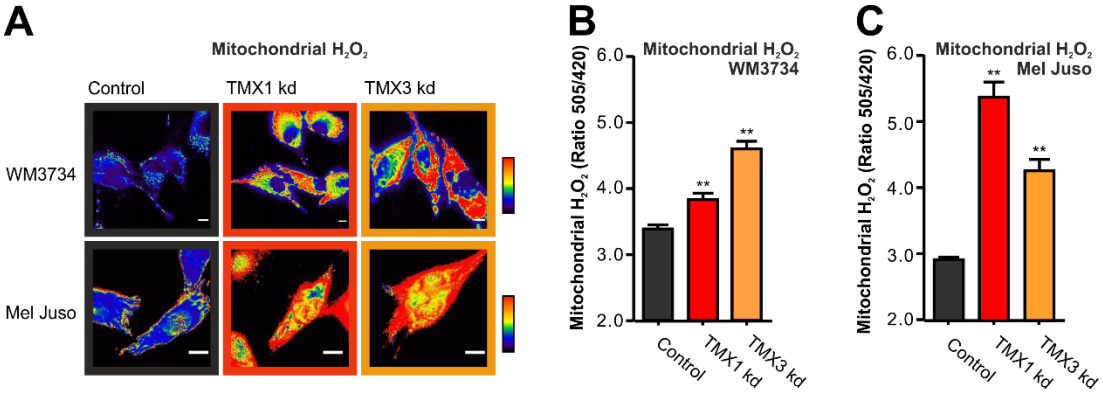


Figure 5A, B and C: Mitochondrial H_2O_2 is increased following TMX1 or TMX3 silencing

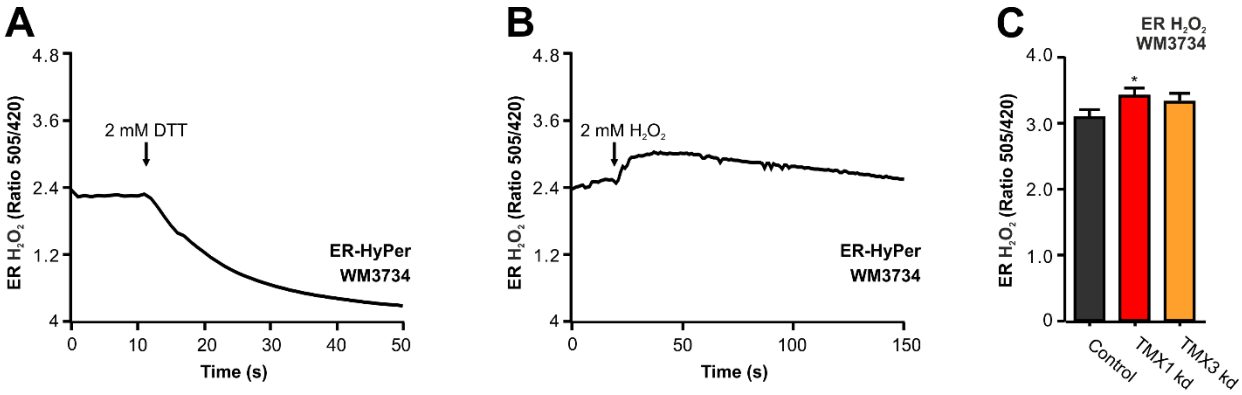


Figure EV4A, B and C: ER H_2O_2 is increased following TMX1 silencing

4. Fig 4. Tapsigargin induced ROS production in these cells ? What are the levels of ROS among the different melanoma cell lines used in these work ? Is there a correlation between the levels of TMX1 and redox state in all cell lines tested ?

We agree that it is important to evaluate the acute effects of thapsigargin on cellular ROS production. Our new results show that thapsigargin does not induce significant changes in global ROS levels (please see Figure EV3H above and our response to reviewer 2, point 5). Determining the general redox state of all melanoma lines and its correlation with TMX1/3 abundance is not trivial. This is because small differences might be difficult to detect due to the sensitivity of the currently available ROS-detecting probes/sensors. Moreover, the resting ROS levels in cells are determined not only by the ER-mitochondrial communication, but also by other parameters such as metabolic state, mitochondrial density, abundance of antioxidant and pro-oxidant enzymes and molecules, etc. Nevertheless, we have performed HyPer-based H_2O_2 measurements and correlated the H_2O_2 levels with the TMX1 expression (qPCR). Indeed, the data shown in Fig. EV3I and J suggest a possible link between TMX1 expression and intracellular H_2O_2, thus supporting the important role of the TMX oxidoreductases as regulators of the cellular redox status.

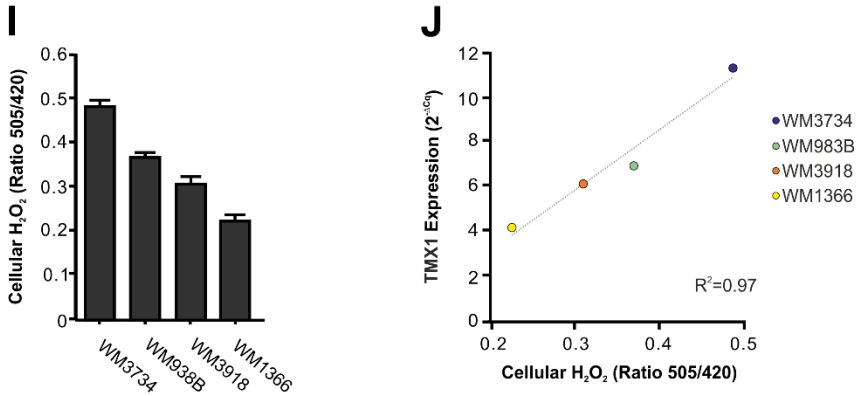


Figure EV3I and J: TMX1 expression levels control cellular redox state

5. Figure 5. The connection between ROS and NFAT translocation is weak. A molecular mechanism should be proposed and/or tested. What' s the molecular relationship between NOX4 and TMX ? Is this just epiphenomena or there is a molecular link ?

Our results show that TMX silencing-induced ROS oxidize and thus inactivate calcineurin, a phosphatase responsible for dephosphorylating NFAT1 and thereby its nuclear translocation. Figure 4 highlights the role of calcineurin as a molecular link between ER-mitochondria communication, ROS and NFAT1.

A recently published paper identified TMX as one of the strongest interaction partners of NOX4 (J Biol Chem. 2016 Mar 25;291(13):7045-59). Exploring the molecular details of this interaction would be very interesting, but also a huge effort and out of the scope of this study.

6. Figure 6: Mild phenotypes are achieved both in vitro and in vivo. In vitro migration assays can not recapitulate tumor invasion phenotype. Also, the in vivo exp are just xenograft and are not measuring invasion.

The bioinformatic analyses of melanoma patient data (TCGA) suggest that NFAT1 and TMX1/TMX3 determine patient survival probability and thus indicate that these proteins are involved in defining melanoma aggressive behavior. The IHC patient data and the *in vitro* experiments support the important role of NFAT1 and TMX in melanoma progression. Indeed, transwell migration is only one way to determine invasive potential. As mentioned above, our mouse studies are secondary to and only support the more important patient derived data. Nevertheless, to address this issue in more detail, we performed additional invasion assays. The new results shown in Fig. 6H show that TMX1 knockdown cells have decreased invasive potential when compared to the control transfected cells.

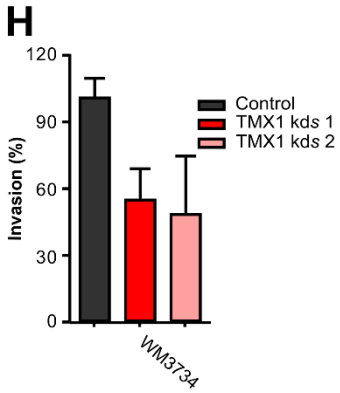


Figure 6H: TMX1 silencing inhibits transwell invasion of WM3734 melanoma cells

7. I don't see the important and specific role of TMX3 here. Authors mentioned the separate role of TMX3 as a prognostic marker in the table S2, but that's it. Usually,

they described the role of TMX3 in accordance with TMX1 in some experiments. Is it redundant compared to TMX1?.
The role of TMX3 was studied in almost all experiments where the role of TMX1 was also examined and results show that they play similar roles, at least under the experimental conditions applied in this study. However, TMX3 as a prognostic marker appears to have additional properties in patients and we believe this is an interesting scientific question to explore in the future. Given that currently very little is known about the functional role of TMX3, we would like to keep the TMX3 data in the paper and highlight its potential for future study.

8. Not sure that 2 samples for IHC were statistically significant: it might better to have 3 samples for each group.
The focus of this figure was to show that NFAT1 and TMX1 increase with melanoma stage. Currently, we have six different conditions from 13 patients showing increased expression with aggressive disease. We are not sure what the reviewer means by "two samples". For the most relevant conditions, i.e. melanoma with tumor thickness lower than 2 mm, melanoma with tumor thickness higher than 4 mm and metastatic melanoma, we show data from at least three patients.

9. I am not certain that is correct to use data from experiments with HeLa cells (fig. 5I-J) to show an increase in mitochondrial exposure to the PM. These figures are representative, but it is not melanoma.
We agree that it was important to evaluate mitochondrial positioning in melanoma cells as well. We have used electron microscopy as well as confocal microscopy of melanoma cells to evaluate the mitochondrial morphology and positioning following TMX1 knockdown. Please see new data shown in Fig. 5I-N and our response to reviewer 2 point 6.

10. I suggest to place the section «Fura-based Ca2+-imaging» under the section «Fluorescence microscopy» in Materials and Methods.
We agree. However, given that we used different microscope setups for measuring Fura-2, protein-based sensors and mitochondrial volume and surface, this is unfortunately not feasible. In order not to confuse the reader we described these approaches separately in the methods section. To make the use of different microscope setups more obvious, the sub-headings under the fluorescence microscopy heading are now reformatted.

11. I suggest to write the section «Statistical analysis» in Materials and Methods.
We agree. Such a section has now been provided in the section "Data and statistical analysis".

12. It is better to mark molecular weight on all Western blot figures.
This has now been done as suggested.

Minor suggestions/questions:
We thank the reviewer for identifying these minor issues and for her/his suggestions. We addressed/corrected the figures and the text as suggested.

13. Page 8 section «NFAT nuclear translocation is impaired in TMX-silenced melanoma cells»: it is better to explain and provide a rationale why these melanoma cell lines were selected in the text. Also, Fig 1E should be moved to Supplementary information and enlarged. It is difficult to identify cellular information at this resolution.
Explanation and rationale regarding the melanoma cells used in this study has been provided (please see Results section). We also provide high-resolution images, which allow better cellular information.

14. Fig S1A/B lacks statistical analyses.
Corrected and additional new data sets included.

15. Pag. 9. I would remove TMX3 from the title of the results section.
As discussed above, we would rather keep TMX3 in the study.

16. Page 10, 2nd paragraph, link to the Fig4H-I in the text: I think authors could mention that these data obtained from MelJuso cells,
Done.

17. Page 12, paragraph 1st, link to the fig. 5I-J: they should mention that is HeLa cell line
Done.

18. Page 14, paragraph 1st: authors did not show that TMX1 knockdown is stable, might be that is why tumor growth is stopped to grow for a while,
This is shown, please see Fig. EV5D.

19. Page 23, section «Cell culture and reagents»: might be it is better to mention that all cell lines were checked for their cellular identity with appropriate markers (see journal policy).
Done.

20. Page 23, section «Cell culture and reagents», 12th line: «and» is repeated twice,
Corrected.

21. Page 24, section «Fura-based imaging»: more details about the microscope, camera, objective and how Ca2+ was evaluated,
This information is provided.

22. Page 25, section «Fluorescence microscopy»: more details of the microscope, conditions of imaging: temperature, CO2...
Done.

23. Page 25, section «Ca2+ and hydrogen peroxide measurements»: how FRET was measured, equation,
Done.

24. Page 26, 2nd paragraph: how the protein was extracted, more details of phosphatase activity measurement,
Done.

25. Page 27, section RT-qPCR: how was mRNA isolated and thermo-cycling conditions,
Information about RT-qPCR is provided in the methods section.

26. Page 27, section «Immunoblotting»: dilution of antibodies is not written,
The antibody dilutions are now added in Appendix Table S7.

27. Page 27, section «Determination of mitochondrial value and surface»: which cells? how were determined volume and surface, formula?
This information is now provided.

28. Page 29, section «Immunohistochemistry»: how images were acquired?
This information is now provided.
"Photographs were taken with an Axio Imager M1 and recorded using the Axiovision software Rel 4.7 (Zeiss, Göttingen, Germany)."

29. Page 29, section «In vivo studies»: how many mice were in each group, gender and age, at which size tumors were fixed?
This information has now been added. Male mice (8 weeks of age, n=7 mice/group), acquired tumors up to 15 mm in diameter prior to sacrifice.

30. Page 38, Fig 1C: I think it is better to do western for all 10 melanoma cells with quantification of density, especially for TMX1,
We have now added new western blots from additional cell lines and performed band quantitation. See Figure 1C above.

31. Page 46, Fig 7A: GOI could be deciphered,
Done.

32. Page 52, Fig S5B: GAPDH bands are saturated too much, there is no space below the bands in blots of Mel Juso and SK Mel5; it not clear which band to analyse on BiP blot for Mel Juso cells (there are 3 bands),

The blots in Fig EV4E have now been rearranged to be easier to interpret. We would like to mention that these experiments were used only to examine if TMX silencing causes ER stress. Given that we do not have any indication of ER stress following TMX1 or TMX3 silencing and the fact that some of these antibodies are not commercially available, we would rather keep these data in the current figure.

33. Page 54, Fig. S6 F-G: pAkt and Akt bands are not similar in the bandwidth and the horizon, and spots, they could be 2 different membranes, but there is 1 control.
Samples in this figure (our new Fig. EV5E) are derived from mouse tumor lysates while samples in Fig. EV5F are from *in vitro* grown cells, so they will not look the same; we now indicate this more clearly in the Figures.
In the first submission, "C4" was shown twice to indicate reproducibility (especially given the known tumor heterogeneity of melanoma); however, we agree that this is more confusing than helpful and we now simplified the Figure.

2nd Editorial Decision 29th Apr 2019

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. Your revised study was sent back to the three referees for re-evaluation, and we have received comments from all of them, which I enclose below. As you will see the referees find that their concerns have been sufficiently addressed and they are now broadly in favour of publication.

We notice that referee #3 states persistent reservations regarding the in vivo relevance of your results. However, in light of the support from the two other referees as well as our editorial assessment of your amended manuscript, we have now concluded that a pathophysiological role of the proposed pathway is sufficiently well supported by the current data.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues related to formatting and data representation, which need to be adjusted at re-submission.

Referee #1:

The authors have greatly improved the manuscript. The unresolved issue of BRAF's role in the NFAT/TMX axis has been clearly investigated. In my opinion, this excellent work deserves to be published and will be of great interest to EMBO readers. It increases knowledge about the role of ROS in the tumorigenesis of melanocytes and precisely describes the role of new actors in this process.

Referee #2:

The authors have satisfactorily replied to my concerns. The manuscript is greatly improved.

Referee #3:

The authors answered to my minor and most major concerns. In Figure 8, the color code for the various cellular component is not clear. I would reformat in a clear manner this graphical

abstract/conclusions.

I'm still concern about the in vivo pathophysiological relevance of these molecular findings and, therefore, the role of TMX-ROS-NFAT1 contribution to aggressive diseases such as melanoma. In this revised version, the authors did not improve the manuscript providing new data and/or evidence on this matter.

2nd Revision - authors' response 21st May 2019

The authors performed the requested editorial changes.

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: Ivan Bogeski
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2018-100871

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

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

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

USEFUL LINKS FOR COMPLETING THIS FORM

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

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
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http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

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

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

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?	For the cell line-based in vitro experiments, at least three biological replicates were used to ensure effective sample size for each experimental condition. The sample size of the in vivo experiment suffice to the criterion of resource equation approach for sample size calculation.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For the animal experiments with WM3734 and the WM1366 cells, 7 mice (21 in total) were used for each experimental group.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We did not exclude samples or animals from the analysis.
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.	Yes. The mice were randomized for allocation of the experimental groups.
For animal studies, include a statement about randomization even if no randomization was used.	The 8-week old male immune deficient NSG mice with similar age were randomized and blindly divided into 3 groups for the experiment.
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.	Blinding of the investigator was used for group allocation of the in vivo xenograft experiments. Blinding of the investigator was also used for the microscopy-based evaluation of mitochondrial positioning.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding of the investigator was used for the group allocation, tumor size measurement, isolation and weighing of the xenograft in the in vivo xenograft experiments.
5. For every figure, are statistical tests justified as appropriate?	Yes. See methods section and figure legends for details.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	Yes, the data are presented as mean±SEM.
Is the variance similar between the groups that are being statistically compared?	Yes. For most of the comparisons, the variance is similar i.e. the sample variance is not larger than twice the size of the other.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The detailed profile of commercially available antibodies used in this study was included under "Materials and Methods" section and in Table S7, the confirmation of proper use was provided by the supplier.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Please refer to the "Materials and Methods" section. The commercially available cell lines were authenticated by the suppliers. The cellular genotypes and cell line identities of melanoma cell lines gifted by Meenhard Herlyn (The Wistar Institute, Philadelphia, USA) were confirmed by DNA fingerprinting, using Geneil's microsatellite kit. All cell lines were tested for mycoplasma

* 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.	Please refer to the "Materials and Methods" section for details. Briefly, male athymic nude mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG), 8 weeks old, Charles River Laboratories, Sulzfeld, Germany) were kept in groups in isolated ventilated cages under specific pathogen-free conditions in a temperature- and humidity-controlled 12 hour dark/light environment at the animal care facility of the Institute for Clinical and Experimental Surgery at Saarland University. Animals had free access to tap water and standard pellet food and their health status was monitored daily.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All mouse experiments were approved by the local governmental animal care committee (Landesamt für Verbraucherschutz des Saarlandes) and were conducted in accordance with the German legislation on protection of animals and the National Institutes of Health Guide for the Care und Use of Laboratory Animals (NIH Publication #85–23 Rev. 1985).
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.	We confirm compliance with the ARRIVE guidelines and NIH and MRC recommendations.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The processing of patient derived materials was performed according to the Goettingen ethics committee votum No. 13/5/17 and the Statement of the National Ethics Council On Biobanks for Research, Berlin, Germany.
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.	The authors state that the human melanoma samples were collected from patients with signed informed consent. The experiments were performed according to the principles of the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	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.	NA