EMBO reports

p38 MAPK-dependent phosphorylation of TFEB promotes monocyte to macrophage differentiation

José A. Martina, Eutteum Jeong and Rosa Puertollano
DOI: 10.15252/embr.202255472

Corresponding author(s): Rosa Puertollano (puertolr@nhlbi.nih.gov)

Review Timeline:

Submission Date:	23rd May 22
Editorial Decision:	28th Jun 22
Revision Received:	22nd Sep 22
Editorial Decision:	7th Nov 22
Revision Received:	14th Nov 22
Accepted:	21st Nov 22

Editor: Martina Rembold

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.)

Dear Dr. Puertollano

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also point out several technical concerns and have a number of suggestions for how the study should be strengthened. The recapitulation of key findings using primary cell models would certainly strengthen the results (referee 2, point A) and should be performed. If available, correlative data on TFEB S401 phosphorylation obtained from mouse disease models would be a valid extension but are not absolutely essential for publication here.

I think it would be helpful to discuss the raised points further and I am available to do so via email or video.

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

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 (September 28, 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

IMPORTANT NOTE:
We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.

2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

When submitting your revised manuscript, we will require:

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).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

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

4) a complete author checklist, which you can download from our author guidelines (). 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 (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. 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 labeled 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.

7) Please structure the Data availability section following the model below (see also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).

Please note that the Data Availability Section is restricted to new primary data that are part of this study.

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

Data availability

The datasets produced in this study are available in the following databases:

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

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

8) Figure legends and data quantification:
The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please also include scale bars in all microscopy images.

9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should 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 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 .

10) 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 .

11) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have

chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

****

Referee #1:

This paper entitled "p38 MAPK-dependent phosphorylation of TFEB is required for efficient monocyhte to macrophage differentiation" by Martina JA et al. shows phosphorylation of S401 by p38 modulates monocyte function and differentiation. Extensive experimental data supports the authors' claim. I have a couple of inquiries.

1. NaAsO2 was employed to impose oxidative stress. It is well known that arsenate is an ER stress inducer. Can other ER stress inducers induce S401-TFEB phosphorylation?
2. In Fig 1H, I cannot find data using R245-247>A.
3. In the last line of p8, is Fig 2B a mistake of Fig 2D?
4. While the authors claim that monocyte differentiation into macrophages are defective in S401 mutant, is there evidence suggesting defective macrophage differentiation such as changes of surface markers? Are the decreased cytokine mRNA levels enough to conclude that macrophage differentiation is defective?
5. In Fig EV6, nuclear translocation of p65 alone was used as a marker of NF-kB activation. Other markers of NF-kB need to be studied.
6. What is the mechanism of defective inflammasome activation by S401 mutation? There are papers reporting that PMA-stimulated THP-1 cells activate inflammasome with LPS alone (Netea M et al. Blood 113:2324, 2009). Are the authors' data consistent with those data?

Referee #2:

The report by Martina and colleagues contributes to our understanding of immune signaling by showing that phosphorylation of S401 in TFEB regulates cellular responses to oxidative stress, UVC, and LPS in a p38 MAPK-dependent fashion. In addition, the report seems to support the claim that this phosphorylation event plays a critical role during monocyte differentiation into macrophage-like cells. Based on these findings, the authors conclude that TFEB-S401 phosphorylation "links differentiation signals to the transcriptional control of monocyte differentiation."

Strengths of the report include (a) the use of site-specific mutations to clarify the cell biological role of S401 phosphorylation; (b) high-quality images and western blots that support the conclusions of the manuscript; (c) mutually supportive data derived from the analysis of cellular responses at the RNA and protein levels; (d) statistical rigor.

Despite these strengths, the manuscript suffers from several weaknesses that must be addressed to fully support the overarching conclusions.

(A) Absence of primary cell models to verify findings (Major Weakness)
The report appears to exclusively use immortalized cell models of monocytes and macrophages. While RAW264.7 and THP1 cells provide useful systems for analyzing immune functions, the recapitulation of these findings in primary cell models are required to enhance the overall rigor and further support the conclusions of these studies.

(B) Absence of in vivo support of key findings (Minor Weakness)
The report would be enhanced by the inclusion of data from in vivo models of disease which demonstrate that the identified phosphorylation event occurs in diseased (but not in disease-free) animals. A wide variety of animal models could be used to support such findings, and the inclusion of such correlative data would significantly strengthen the narrative.

Referee #3:

Although many kinases are known to phosphorylate TFEB and regulate TFEB transcription activity, in this study, the authors have identified S401 as a novel phosphorylation site, which is dependent on p38 activation. They showed that phosphorylation at S401 does not affect the nuclear translocation of TFEB and the transcription of known TFEB target genes under cell stress, while they suggested that this phosphorylation is required for TFEB nuclear translocation and activation during monocyte differentiation. Their findings are unique and interesting. However, there are several core questions that need to be answered before this paper can be considered for publication.

Major points
1. It is not clear if p38 directly phosphorylates TFEB at S401. Protein interaction assay and kinase assay are required to determine this. The results will help to clarify the relevant ambiguous issues.
2. Because mTORC1-mediated phosphorylation at S211 predominantly determines the distribution of TFEB. A key issue is the relationship between p-S211 and p-S401. Based on the immunoblot data, there were two bands of TFEB in gel, and p-S211 is mainly contained in the upper band while p-S401 is mainly included in the lower one. It seems that phosphorylation at S401 is linked to the dephosphorylation at S211. To determine if phosphorylation at S401 can sufficiently cause TFEB nuclear translocation, it would be interesting to see the distribution of S211A-S401D mutant in ARPE-19 or U2OS cells.
3. The authors claimed that the reduced nuclear accumulation of TFEB-S401A in undifferentiated monocytes but not differentiated macrophages and ARPE-19 cells is due to the degradation of TFEB-S401A in the nucleus or possible interaction with other proteins. In either case, the proteasomal inhibitor should to used and the binding capacity of TFEB-S401A vs WT TFEB to related gene promoters should be checked to clarify this.
4. Under the same scenario, it is interesting that in PMA-treated THP1 cells, phosphorylation at S401 is only required for the expression of immune genes but not autophagy and lysosomal genes. To confirm it and clarify the potential mechanism, the authors can compare the binding affinity of TFEB-S211A vs TFEB-S401D to the promoter of the two groups of genes.

Minor
There are many errors in the References, including initials and page numbers.

Referee #1:

This paper entitled "p38 MAPK-dependent phosphorylation of TFEB is required for efficient monocyte to macrophage differentiation" by Martina JA et al. shows phosphorylation of S401 by p38 modulates monocyte function and differentiation. Extensive experimental data supports the authors' claim. I have a couple of inquiries.

1. NaAsO2 was employed to impose oxidative stress. It is well known that arsenate is an ER stress inducer. Can other ER stress inducers induce S401-TFEB phosphorylation?

To address the reviewer's question, we treated HeLa-TFEB-Flag cells with either tunicamycin or thapsigargin, two well-known ER stress inducers, for 1h and 6h. As seen in **Appendix Figure S1A**, both compounds induced ER stress efficiently, as assessed by the increase in ATF4 expression. In addition, induction the ATF4 target CHOP was observed after 6h treatment. However, neither compound induced p38 activation or TFEB phosphorylation at S401, suggesting that ER stress is not sufficient to cause S401-TFEB phosphorylation. In contrast, incubation with $NaAsO_2$ clearly induced p38 activation and S401-TFEB phosphorylation (**Appendix Figure S1A**). It is important to note that we did not observe a clear induction of ER stress at the $NaAsO_2$ concentration and treatment time used in these experiments (200 μM $NaAsO_2$ for 1h), further supporting the idea that S401-TFEB phosphorylation occurs independently of ER stress. Three independent experiments were performed, and their quantification is shown in **Appendix Figure S1B**.

2. In Fig 1H, I cannot find data using R245-247>A.

We apologize if this point was not made sufficiently clear in the previous version of the manuscript. The R245-247>A mutant was showed in the figure as *Δ*NLS to indicate that we mutated the nuclear localization signal (NLS), and this was mentioned in the corresponding figure legend. To avoid confusion, we have now labeled the mutant as R245-247A in **Figures 1G and 1H**.

3. In the last line of p8, is Fig 2B a mistake of Fig 2D?

We apologize for the mistake, instead Fig 2B, it should read **Fig EV2B**. This has been corrected.

4. While the authors claim that monocyte differentiation into macrophages are defective in S401 mutant, is there evidence suggesting defective macrophage differentiation such as changes of surface markers? Are the decreased cytokine mRNA levels enough to conclude that macrophage differentiation is defective?

Whereas we agree with the reviewer in that monocyte differentiation is not completely blocked in mutant cells, our data demonstrate that it is indeed defective. We based our conclusion in several points of evidence. First, our RNA-seq analysis clearly shows that immune and inflammatory genes are the most significantly induced following PMA treatment (**Figure 5C**), suggesting that this upregulation, which is severely reduced in mutant cells, is a critical component of monocyte differentiation. Second, we did observe abnormal expression of some surface markers. For example, we show that the expression of CD14 is significantly diminished in mutant cells (**Figure EV5D**). Finally, the inability of the mutant macrophages to acquire M1 inflammatory phenotypes even at early times of LPS stimulation, further suggests that monocytes differentiation into M0 macrophages was abnormal.

5. In Fig EV6, nuclear translocation of p65 alone was used as a marker of NF-kB activation. Other markers of NF-kB need to be studied.

As requested by the reviewer, we further analyzed NF-kB activation by comparing phosphorylation of IκBα in THP1-WT and THP1-I11 cells in response to LPS. It is well established that phosphorylation of IκBα followed by proteasome-mediated degradation results in the release and nuclear translocation of active NF-κB. Accordingly, we observed a robust increase in IκBα phosphorylation in differentiated THP1 macrophages after 1h incubation with LPS, together with a concomitant reduction in IκBα levels (**Appendix Figure S2A**). No significant differences were found between WT and I11 cells, further suggesting that NF-κB activation is not affected in TFEB-S401A-expressing cells (**Appendix Figure S2B**).

6. What is the mechanism of defective inflammasome activation by S401 mutation? There are papers reporting that PMA-stimulated THP-1 cells activate inflammasome with LPS alone (Netea M et al. Blood 113:2324, 2009). Are the authors' data consistent with those data?

In agreement with previous studies, we detected a small increase in inflammasome activation in PMA-stimulated THP-1 cells treated with LPS alone, as assessed by increased levels of mature IL-1β (**Figure 6B and 6D**), GSGMD cleavage (**Figure 6B and 6F**), and number of cells with Asc specks (**Figure EV6F**). However, this activation is modest when compared with cells treated with both, LPS and nigericin (**Figures 6B, 6D, 6F, and EV6F**).

Referee #2:

The report by Martina and colleagues contributes to our understanding of immune signaling by showing that phosphorylation of S401 in TFEB regulates cellular responses to oxidative stress, UVC, and LPS in a p38 MAPK-dependent fashion. In addition, the report seems to support the claim that this phosphorylation event plays a critical role during monocyte differentiation into macrophage-like cells. Based on these findings, the authors conclude that TFEB-S401 phosphorylation "links differentiation signals to the transcriptional control of monocyte differentiation."

Strengths of the report include (a) the use of site-specific mutations to clarify the cell biological role of S401 phosphorylation; (b) high-quality images and western blots that support the conclusions of the manuscript; (c) mutually supportive data derived from the analysis of cellular responses at the RNA and protein levels; (d) statistical rigor.

We thank the reviewer for the support

Despite these strengths, the manuscript suffers from several weaknesses that must be addressed to fully support the overarching conclusions.

(A) Absence of primary cell models to verify findings (Major Weakness)
The report appears to exclusively use immortalized cell models of monocytes and macrophages. While RAW264.7 and THP1 cells provide useful systems for analyzing immune functions, the recapitulation of these findings in primary cell models are required to enhance the overall rigor and further support the conclusions of these studies.

Please see below our response to comments (A) and (B) together.

(B) Absence of in vivo support of key findings (Minor Weakness)
The report would be enhanced by the inclusion of data from in vivo models of disease which demonstrate that the identified phosphorylation event occurs in diseased (but not in disease-free) animals. A wide variety of animal models could be used to support such findings, and the inclusion of such correlative data would significantly strengthen the narrative.

We agree with the reviewer in that it would be informative to confirm our conclusions in animal models. However, this would require the generation of knock-in mice carrying a serine to alanine mutation in residue 401 and as such, it would demand a big commitment in both time and resources. Confirmation of some of our key results in primary cells is also important but we have the caveat that our anti-S401 phospho-specific antibody does not recognizes mouse TFEB, thus preventing us from using BMDMs and limiting our options.

To address the reviewer's concerns, we isolated undifferentiated monocytes from human blood and differentiated them into macrophages by incubation with 25 ng/ml GM-CSF for a total of 6 days (**Figure EV3A**). Differentiation of monocytes into macrophages was assessed by monitoring the expected decreased in CD14 and CD16 levels, together with the simultaneous increase in CD68 and CD71 (**Figure EV3B**). In agreement with our results in Raw 264.7 and THP1 cells, we detected p38 activation and increased TFEB-S401 phosphorylation in response to LPS in human macrophages (**Figure EV3C and EV3D**), thus confirming our main observation in primary cells.

Referee #3:

Although many kinases are known to phosphorylate TFEB and regulate TFEB transcription activity, in this study, the authors have identified S401 as a novel phosphorylation site, which is dependent on p38 activation. They showed that phosphorylation at S401 does not affect the nuclear translocation of TFEB and the transcription of known TFEB target genes under cell stress, while they suggested that this phosphorylation is required for TFEB nuclear translocation and activation during monocyte differentiation. Their findings are unique and interesting. However, there are several core questions that need to be answered before this paper can be considered for publication.

Major points
1. It is not clear if p38 directly phosphorylates TFEB at S401. Protein interaction assay and kinase assay are required to determine this. The results will help to clarify the relevant ambiguous issues.

We thank the reviewer for this suggestion. It is well-stablished that p38 can phosphorylate and activate several kinases, which in turn phosphorylate additional proteins to regulate a wide variety of processes (Canovas and Nebreda, 2021). Of relevance are MSK1 and MSK2, which phosphorylate transcription factors implicated in immune response and differentiation. Additional kinases that are regulated by p38 include MAPKAPK2, MAPKAPK3, MNK1 and MNK2. We used specific siRNAs and inhibitors to assess the potential contribution of p38-regulated kinases to TFEB-S401 phosphorylation. As seen in **Figure EV2E and EV2F**, neither depletion of MSK1, MSK2, MAPKAPK2 and MAPKAPK3, nor inhibition of MNK1 and MNK2, prevented increased S401 phosphorylation in response to $NaAsO_2$, suggesting that p38 MAPK likely phosphorylate TFEB directly. To further confirm this, we performed *in vitro* kinase assays. TFEB C-terminal

region, which includes the proline-rich domain, was fused to GST, purified, and incubated with recombinant active p38α MAPK. As expected, phosphorylation of TFEB-S401 was detected only when both p38 MAPK and ATP were present (**Figure 2F and 2G**). These results provide strong evidence that p38 MAPK directly phosphorylates TFEB at S401.

2. Because mTORC1-mediated phosphorylation at S211 predominantly determines the distribution of TFEB. A key issue is the relationship between p-S211 and p-S401. Based on the immunoblot data, there were two bands of TFEB in gel, and p-S211 is mainly contained in the upper band while p-S401 is mainly included in the lower one. It seems that phosphorylation at S401 is linked to the dephosphorylation at S211. To determine if phosphorylation at S401 can sufficiently cause TFEB nuclear translocation, it would be interesting to see the distribution of S211A-S401D mutant in ARPE-19 or U2OS cells.

As suggested by the reviewer, we investigated in more detail whether the phosphorylation status of S401 may influence phosphorylation of S211 and, consequently, TFEB intracellular distribution and activation. For this, we performed several different experiments. First, we addressed whether S401 phosphorylation may prevent S211 dephosphorylation in response to $NaAsO_2$. In agreement with our previous studies (Martina and Puertollano, 2018), we observed over 50% reduction in the levels of phospho-S211 after 1h treatment with $NaAsO_2$, but this reduction was not affected by mutation of S401 to either alanine (TFEB-S401A) or aspartic acid (TFEB-S401D) (**Appendix Figure S3A and S3B**). Furthermore, mutation of S211 does not prevent phosphorylation of S401 in response to $NaAsO_2$ (**Appendix Figure S3A**). Second, TFEB-S401A and TFEB-S401D translocate to the nucleus with the same efficiency than TFEB-WT (**Appendix Figure S3C and S3D, Figure 1I, Figure EV1C and EV1D**) and display comparable transcriptional activity under over-expression conditions (**Figure EV1E**). Finally, the phosphomimetic mutation of S401 did not prevent the constitutive nuclear accumulation of the S211A mutant (**Appendix Figure S3D and S3E**). Altogether, our data indicate that phosphorylation of S211 and S401 are independent of each other.

3. The authors claimed that the reduced nuclear accumulation of TFEB-S401A in undifferentiated monocytes but not differentiated macrophages and ARPE-19 cells is due to the degradation of TFEB-S401A in the nucleus or possible interaction with other proteins. In either case, the proteasomal inhibitor should be used and the binding capacity of TFEB-S401A vs WT TFEB to related gene promoters should be checked to clarify this.

We thank the reviewer for the suggestion. We have now pre-treated THP1 cells with proteosome inhibitors (MG132) for three hours prior to the addition of PMA. After one hour of PMA treatment, cells were analyzed by subcellular fractionation. Similar to our previous results, we found that the amount of nuclear TFEB-S401A was dramatically reduced when compared with TFEB-WT. Surprisingly, the levels of nuclear TFEB-S401A remained barely detectable, even in the presence of MG132. Increased accumulation of ubiquitinated proteins was used as a read out for the efficiency of the MG132 inhibitor. Furthermore, TFEB-S401 levels were not rescued by treatment with Leupetin+E64d, ruling out the possibility that the mutant is being degraded in lysosomes. Four independent experiments were performed providing similar results. A representative experiment is shown in **Appendix Figure S4**. There are several potential explanations for these results. One possibility is that TFEB-S401 degradation occurs in the nucleus and that the nuclear proteosome is not being efficiently inhibited by MG132 at the concentrations used in these experiments. Alternatively, TFEB-S401 might undergo protease-specific cleavage, which would agree with the presence of proteolytic fragments shown in **Figure 4F**. In agreement with this idea, previous studies have suggested a role of caspases in differentiation of monocytes into

macrophages (Sordet et al., 2002), and caspase-2 and caspase-3 have been found to cleavage proteins in the nucleus (Colussi et al., 1998; Kamada et al., 2005; Maruoka et al., 2021).

4. Under the same scenario, it is interesting that in PMA-treated THP1 cells, phosphorylation at S401 is only required for the expression of immune genes but not autophagy and lysosomal genes. To confirm it and clarify the potential mechanism, the authors can compare the binding affinity of TFEB-S211A vs TFEB-S401D to the promoter of the two groups of genes.

The reviewer raises an interesting point, does phosphorylation of S401 affects the binding affinity of TFEB to the promoter of targets genes? To address this question, we treated THP1-WT and THP1-I11 cells with PMA for 12h and performed ChIP-seq analysis. Our data reveal that TFEB-WT and TFEB-S401A bind to the promoter of lysosomal and immune genes with similar affinity (**Appendix Figure S5A and S5B**), suggesting that phosphorylation of S401 does not prevent TFEB promoter occupancy and that the reduced expression of TFEB targets in mutant cells is likely due to either the reduced nuclear accumulation of TFEB-S401 or the abnormal interaction with potential transcriptional regulators, as suggested in the discussion.

Minor
There are many errors in the References, including initials and page numbers.

This has been corrected

We want to thank all the reviewers one more time for their constructive and valuable comments. All the points raised were quite useful and helped us to improve our manuscript.

REFERENCES

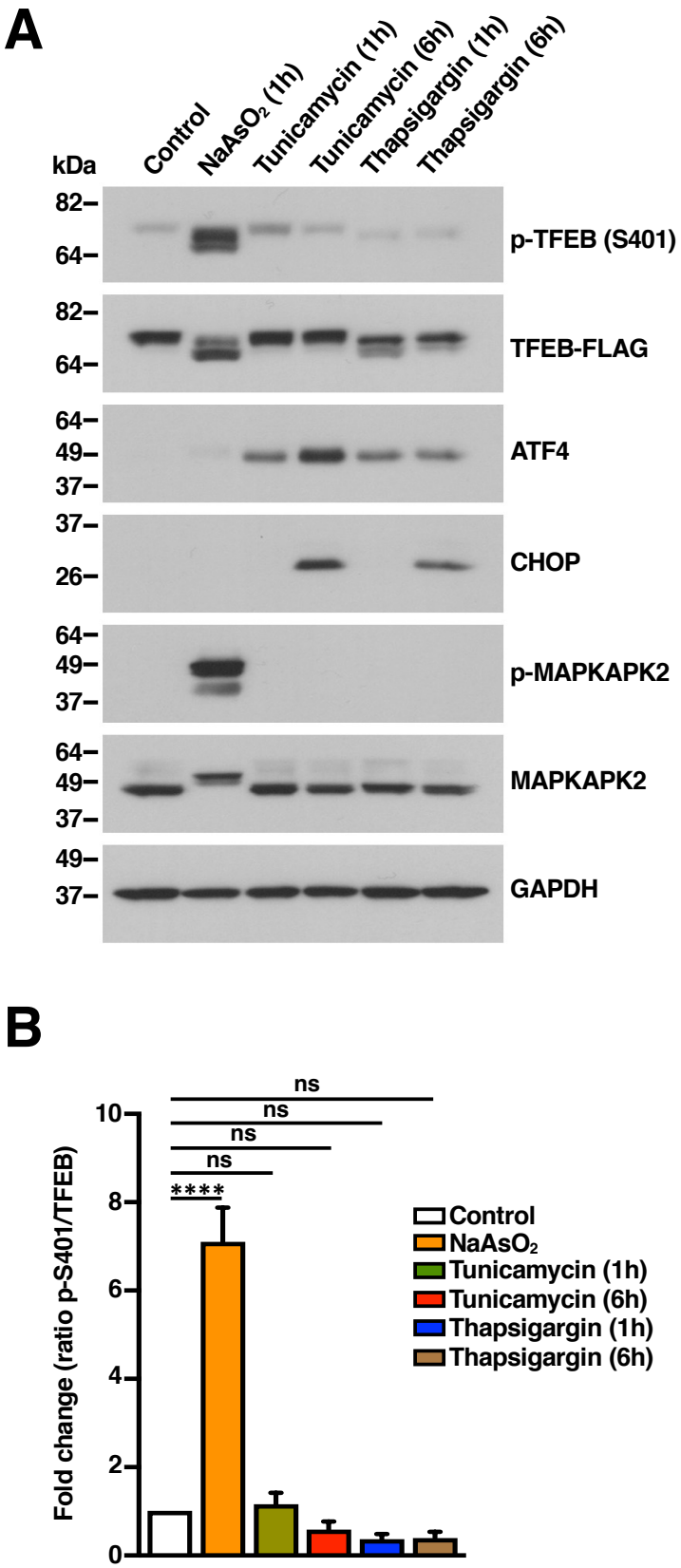
Canovas B, Nebreda AR. Diversity and versatility of p38 kinase signaling in health and disease. Nat Rev Mol Cell Biol. 2021; 22(5):346-366

Colussi PA, Harvey NL, Kumar S. Prodomain-dependent nuclear localization of the caspase-2 (Nedd2) precursor. A novel function for a caspase prodomain. J Biol Chem. 1998; 273(38):24535-42

Kamada S, Kikkawa U, Tsujimoto Y, Hunter T. Nuclear translocation of caspase-3 is dependent on its proteolytic activation and recognition of a substrate-like protein(s). J Biol Chem. 2005; 280(2):857-60

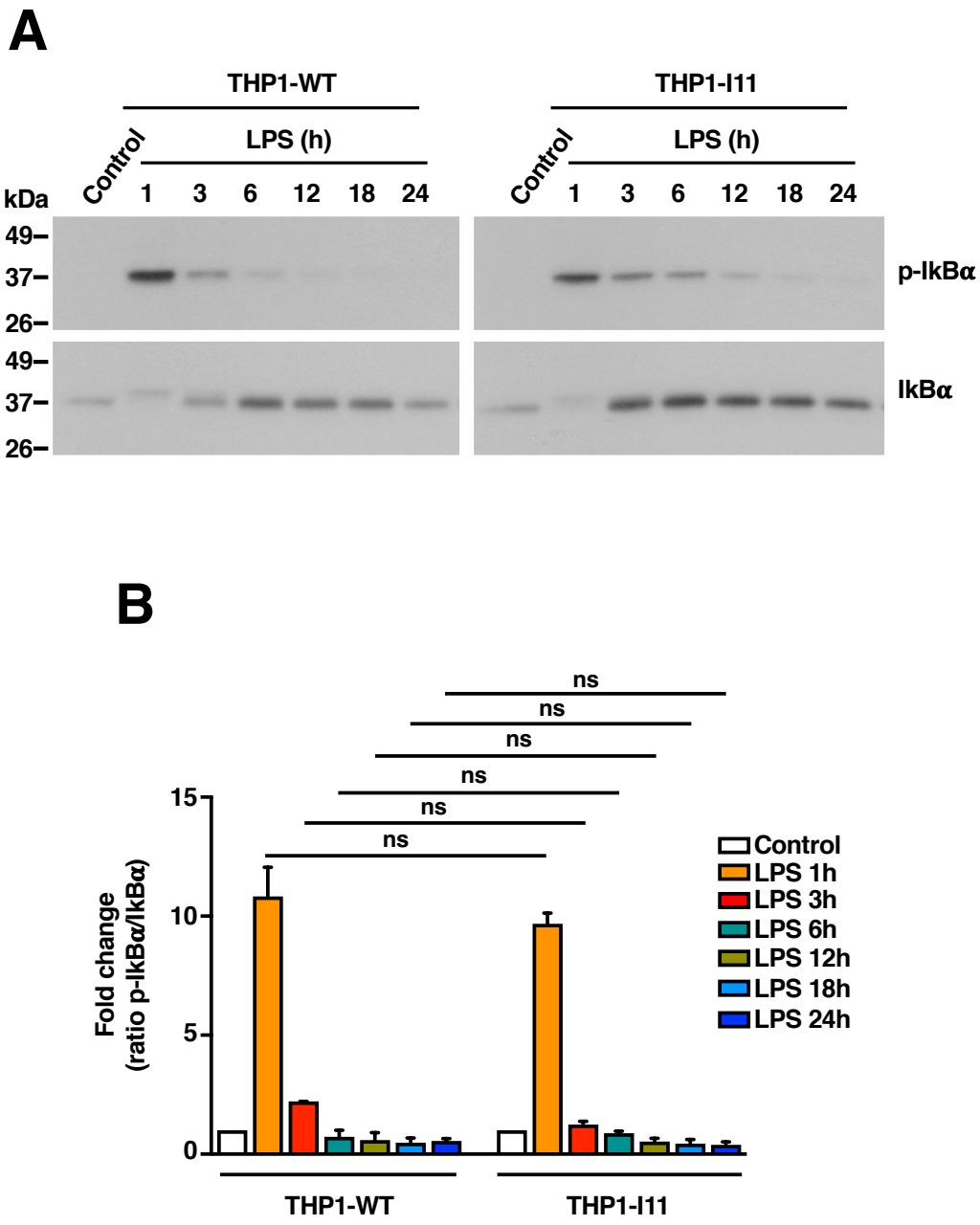
Martina JA, Puertollano R. Protein phosphatase 2A stimulates activation of TFEB and TFE3 transcription factors in response to oxidative stress. J Biol Chem. 2018; 293(32):12525-1253

Maruoka M, Zhang P, Mori H, Imanishi E, Packwood DM, Harada H, Kosako H, Suzuki. Caspase cleavage releases a nuclear protein fragment that stimulates phospholipid scrambling at the plasma membrane. J. Mol Cell. 2021; 81(7):1397-1410.e9



Appendix Figure S1. ER stress is not responsible for TFEB serine 401 phosphorylation upon $NaAsO_2$ treatment.

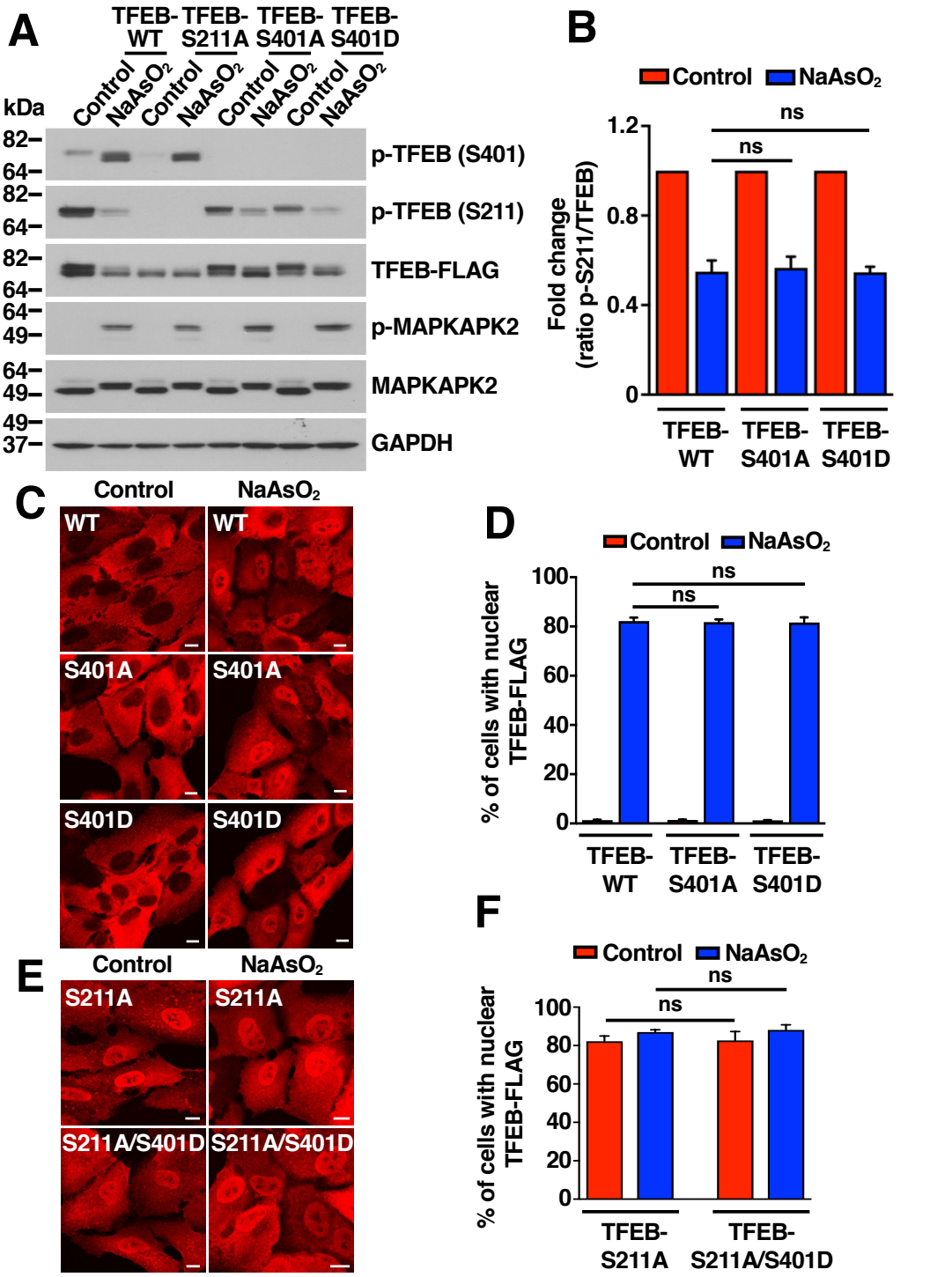
A. Immunoblot analysis of protein lysates from HeLa cells stably expressing TFEB-WT-FLAG incubated with either 200 μM $NaAsO_2$ for 1 h or 5 μg/ml Tunicamycin or 100 nM Thapsigargin for 1h and 6h.
B. Quantification of immunoblot data shown in (A). Data are presented as mean ± SD using one-way ANOVA, (ns) not significant, and (****)p<0.0001 from three independent experiments.



Appendix Figure S2. Normal activation of NF-KB **pathway in THP1 S401A mutant cells.**

A. Immunoblot analysis of protein lysates from PMA-differentiated THP1-WT or TFEB-S401A knock-in (clone I11) cells incubated with 1 μg/ml LPS for the indicated times.

B. Quantification of immunoblot data shown in (A). Data are presented as mean ± SD using one-way ANOVA, (ns) not significant as compared to the same treatment condition in THP1-WT cells from two independent experiments.



Appendix Figure S3. Subcellular distribution of TFEB is not affected in S401 mutants upon $NaAsO_2$ treatment.

A. Immunoblot analysis of protein lysates from ARPE-19 cells expressing TFEB-WT-FLAG, TFEB-S211A-FLAG, TFEB-S401A-FLAG or TFEB-S401D-FLAG treated with 200 µM $NaAsO_2$ for 1 h.

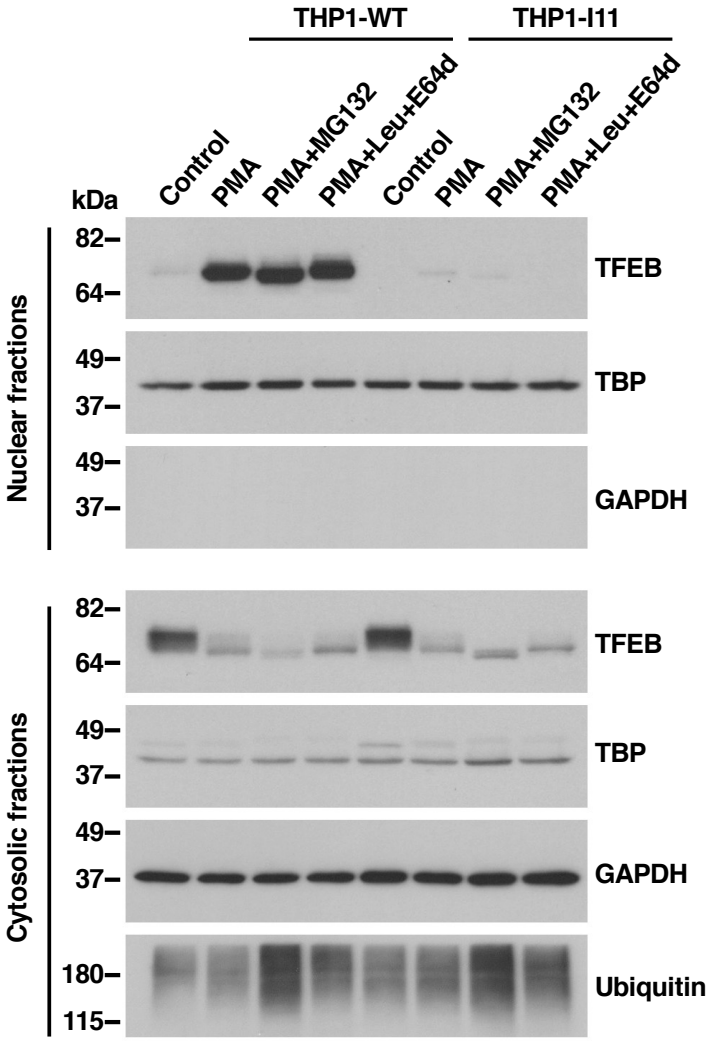
B. Quantification of immunoblot data shown in (A). Data are presented as mean ± SD using one-way ANOVA, (ns) not significant from three independent experiments.

C. Immunofluorescence confocal microscopy of ARPE-19 cells overexpressing TFEB-WT-FLAG, TFEB-S401A-FLAG or TFEB-S401D-FLAG showing the subcellular distribution of recombinant TFEB in response to treatments with 200 µM $NaAsO_2$ for 1 h. Scale bars, 10 µm.

D. Quantification of the nuclear localization of recombinant TFEB in ARPE-19 cells shown in (C). Data are presented as mean ± SD using one-way ANOVA, (ns) not significant as compared to the same treatment in TFEB-WT-FLAG overexpressing cells, with >200 cells counted per trial from three independent experiments.

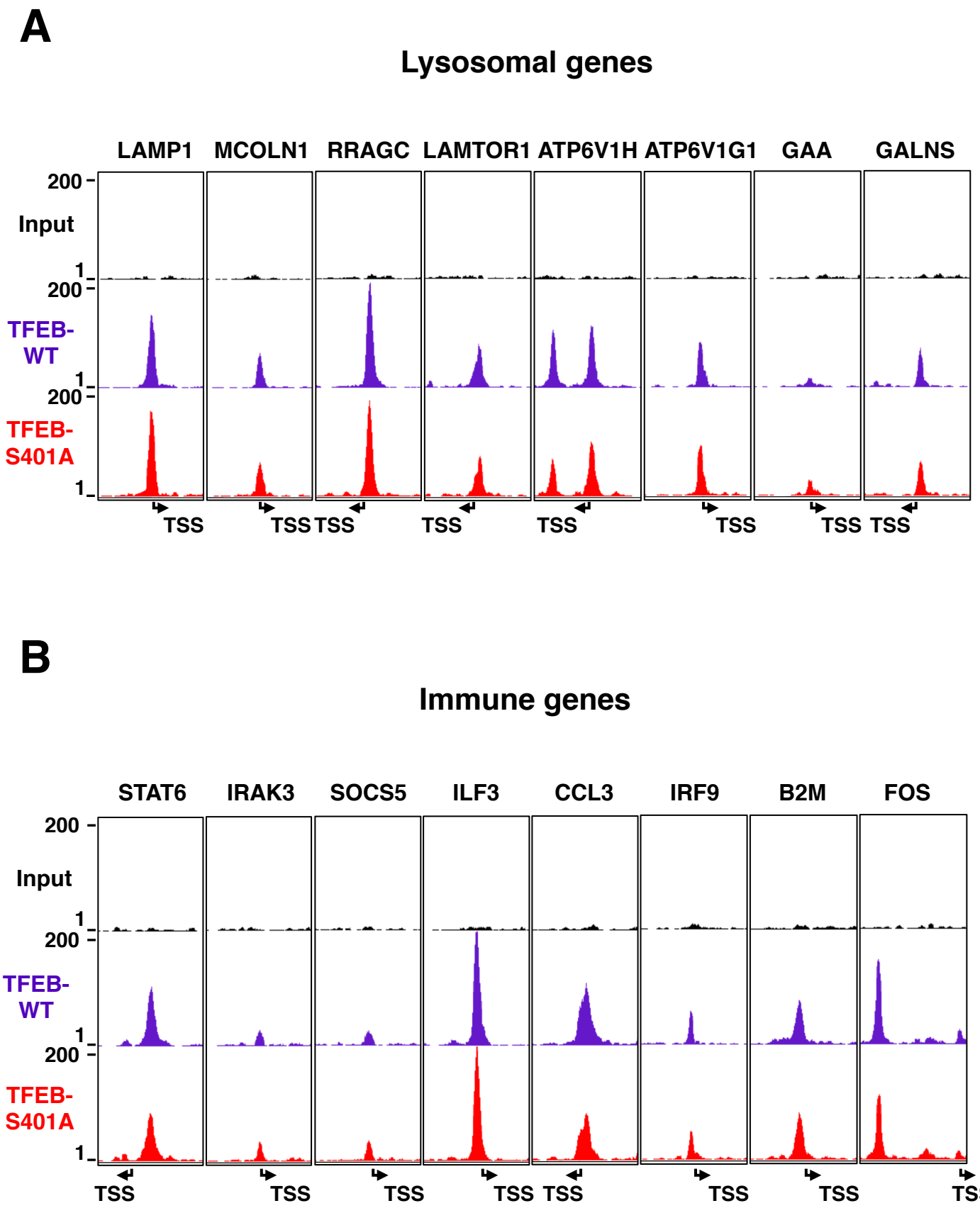
E. Immunofluorescence confocal microscopy of ARPE-19 cells overexpressing TFEB-S211A-FLAG and TFEB-S211A/S401D-FLAG showing the subcellular distribution of recombinant TFEB in response to treatments with 200 µM $NaAsO_2$ for 1 h. Scale bars, 10 µm.

F. Quantification of the nuclear localization of recombinant TFEB in ARPE-19 cells shown in (E). Data are presented as mean ± SD using one-way ANOVA, (ns) not significant as compared to the same treatment in TFEB-S211A-FLAG overexpressing cells, with >150 cells counted per trial from three independent experiments.



Appendix Figure S4. Decreased nuclear accumulation of TFEB-S401A is not caused by proteasomal or lysosomal degradation.

Immunoblot analysis of proteins from nuclear and cytosolic fractions from naïve THP1-WT or TFEB-S401A knock-in (clone I11) cells incubated with either 10 μM GM132 or 100 μg/ml Leupeptin (Leu) and 10 μM E64d for 3 h prior to the addition of 50 ng/ml PMA for 1 h.



Appendix Figure S5. TFEB-WT and TFEB-S401A bind to the promoter of lysosomal and immune genes with similar affinity.

ChIP-seq analysis of lysosomal (**A**) and immune genes (**B**) from THP1-WT and TFEB-S401A knock-in (clone I11) cells treated with 50 ng/ml PMA for 12 h. Arrows indicate the transcription start site (TSS) for each gene analyzed.

Dear Rosa,

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees are very positive about the study and request only minor changes to clarify text and results. Please address the remaining comments from referee 3 in a point-by-point response and in the manuscript text, as you see fit. Points 1 and 2 from referee 1 should be addressed by textual changes and point 3 can be addressed in the final point-by-point response, or mentioned in the text or discussion.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- We can only typeset up to 5 EV figures. Please move one of the EV figures to the Appendix.

- Appendix: please add page numbers to the table of contents.

- Appendix Figure S2B: the data are based on 2 experiments. Therefore, please show the individual datapoints instead of mean and standard deviation and please remove the statistical testing.

- Along these lines: We generally encourage to show the individual datapoints in addition to the mean and error bar in bar graphs if the quantification is based on small sample numbers (i.e., smaller than 5).

- Appendix Figure S5: If I understand correctly, these data are based on a re-analysis of published ChIP-Seq data. I therefore recommend adding a reference of the dataset.

- Appendix Tables S1 & S2 should be called Dataset EV1 & EV2. Their legend needs to be included in a separate sheet of the .xls file.

- Appendix Tables S3-S5 should be added to the Appendix pdf file.

- Please add callouts to the Appendix figures in the text where appropriate.

- Please update the 'Conflict of interest' paragraph to our new 'Disclosure and competing interests statement'. For more information see
https://www.embopress.org/page/journal/14693178/authorguide#conflictsofinterest

- Regarding the Author Contributions, we now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section in the manuscript. Therefore, please remove this section from the manuscript and use the free text box in the submission system instead if you wish to provide more detailed descriptions. See also guide to authors https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines.

- Source data: Please split the file into one file per figure. Source data for EV and Appendix figures should be in a separate folder. You can ZIP everything together and upload the ZIP file.
- The Western blots shown in Fig. 2A have been 'stretched' quite a bit if compared to their format in the source data. I suggest presenting them closer to their original format.

- The EV figure legends should be grouped together after the main figure legends. Please add a heading 'Expanded View Figure Legends'.

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina

Martina Rembold, PhD
Senior Editor

EMBO reports

****

Referee #1:

This revised paper entitled "p38 MAPK-dependent phosphorylation of TFEB is required for efficient monocyte to macrophage differentiation" by Martina JA et al. has been improved by incorporation of the rievewers' comments. I still have a couple of minor inquiries.
1. In Fig EV5D, CD14 data was shown, as stated in the answer to the inquiry 4. However, statement about the difference of CD14 expression could not be found in the text.
2. In Fig. 6, IL-18 release was increased in I11 or M17 cells compared to control cells, while IL-1beta release was decreased in I11 or M17 cells. What is the mechanism of such differences? Such results were not stated or discussed in the text.
3. I could understand that arsenate was used as an ROS inducer and ER stress by arsenate in this investigation was not significant. However, it is still not clear why arsenate was employed as an ROS inducer, while there are many well-known ROS inducers.

Referee #3:

In this revised version, the authors have added in vitro kinase assay and confirmed that p38 can directly phosphorylate TFEB at S401. This is good. However, they did not follow this reviewer's comments to analyze the relationship between S211 phosphorylation and S401 phosphorylation in TFEB nuclear translocation and activation, which I think is important for clarifying the specific effect of S401 phosphorylation on macrophage differentiation.
Nevertheless, I would agree that this revised manuscript can be considered for publication in EMBO Reports.

Referee #1:

This revised paper entitled "p38 MAPK-dependent phosphorylation of TFEB is required for efficient monocyte to macrophage differentiation" by Martina JA et al. has been improved by incorporation of the reviewers' comments.

We thank the reviewer for the support

I still have a couple of minor inquiries.

1. In Fig EV5D, CD14 data was shown, as stated in the answer to the inquiry 4. However, statement about the difference of CD14 expression could not be found in the text.

A statement mentioning the different CD14 expression levels between control and mutant cells has now been included in the text.

2. In Fig. 6, IL-18 release was increased in I11 or M17 cells compared to control cells, while IL-1beta release was decreased in I11 or M17 cells. What is the mechanism of such differences? Such results were not stated or discussed in the text.

At this point we do not have a mechanism to explain the increased pro-IL18 levels in mutant cells. However, since constitutive expression of pro-IL18 is high in monocytes (Gritsenko *et al*, 2020; Puren *et al*, 1999), we hypothesize that increased pro-IL18 levels in mutant cells might constitute an additional indication of defective differentiation.

3. I could understand that arsenate was used as an ROS inducer and ER stress by arsenate in this investigation was not significant. However, it is still not clear why arsenate was employed as an ROS inducer, while there are many well-known ROS inducers.

We agree with the reviewer in that there are many well-known ROS inducers, one of them being $NaAsO_2$. The reason for choosing this particular compound was based on our recent studies in which we showed TFEB and TFE3 activation in response to $NaAsO_2$ both *in vitro* and *in vivo* (*Martina et al., EMBO J, 2021*), characterized a novel PP2A-dependet mechanism of $NaAsO_2$-induced TFEB activation (*Martina and Puertollano, JBC, 2018*), and described a direct role of TFEB in the transcription of multiple antioxidant genes (*Jeong et al., Autophagy, 2022*). Furthermore, we have previously described that the TFE3 nuclear translocation induced by $NaAsO_2$ is prevented by treatment with antioxidants like NAC, suggesting that the generation of ROS is critical for the activation of these transcription factors, and identified specific key residues (such as TFEB-C212 and TFE3-C322) that undergo oxidation in response to this compound (*Martina and Puertollano, JBC, 2018; Martina et al., EMBO J, 2021*).

Referee #3:

In this revised version, the authors have added in vitro kinase assay and confirmed that p38 can directly phosphorylate TFEB at S401. This is good. However, they did not follow this reviewer's comments to analyze the relationship between S211 phosphorylation and S401 phosphorylation in TFEB nuclear translocation and activation, which I think is important for clarifying the specific effect of S401 phosphorylation on macrophage differentiation.

Please note that the relationship between S211 and S401 phosphorylation was extensively addressed in our previous rebuttal letter (**Appendix Figure S1 in the updated version of the manuscript**). We showed that the phosphorylation status of S401 does not influence S211 phosphorylation and vice versa. For example, mutation of S401 to either A or D, did not prevent S211 dephosphorylation or TFEB nuclear translocation in response to stress, while mutation of S211 did not affect S401 phosphorylation. Furthermore, nuclear TFEB is always dephosphorylated at S211 but maintains S401 phosphorylation following p38 activation. It may be important to mention that it is not possible to generate phosphomimic mutations for S211, since mutation of S211 to D reduces 14-3-3 binding (14-3-3 requires a phosphorylated serine residue, not just a negative charge), causing TFEB nuclear translocation.

Nevertheless, I would agree that this revised manuscript can be considered for publication in EMBO Reports.

We thank the reviewer for the support

REFERENCES

Gritsenko A, Yu S, Martin-Sanchez F, Diaz-Del-Olmo I, Nichols EM, Davis DM, Brough D, Lopez-Castejon G (2020) Priming Is Dispensable for NLRP3 Inflammasome Activation in Human Monocytes In Vitro. ***Front Immunol*** **11**: 565924

Puren AJ, Fantuzzi G, Dinarello CA (1999) Gene expression, synthesis, and secretion of interleukin 18 and interleukin 1beta are differentially regulated in human blood mononuclear cells and mouse spleen cells. ***Proc Natl Acad Sci U S A*** **96**: 2256-2261

Martina JA, Puertollano R (2018) Protein phosphatase 2A stimulates activation of TFEB and TFE3 transcription factors in response to oxidative stress. ***J Biol Chem*** **293**: 12525-12534

Jeong E, Martina JA, Contreras PS, Lee J, Puertollano R. (2022) The FACT complex facilitates expression of lysosomal and antioxidant genes through binding to TFEB and TFE3. **Autophagy**. **18**: 2333-2349

Martina JA, Guerrero-Gomez D, Gomez-Orte E, Antonio Barcena J, Cabello J, Miranda-Vizuete A, Puertollano R (2021) A conserved cysteine-based redox mechanism sustains TFEB/HLH-30 activity under persistent stress. ***EMBO J*** **40**: e105793

Dr. Rosa Puertollano
NIH
Laboratory of Cell Biology, National Heart, Lung, and Blood Institute
50 South Drive
Building 50, room 3537
Bethesda 20892
United States

Dear Rosa,

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

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

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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

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

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

Kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

****

THINGS TO DO NOW:

Please note that you will be contacted by Wiley Author Services to complete licensing and payment information. The required 'Page Charges Authorization Form' is available here: https://www.embopress.org/pb-assets/embo-site/er_apc.pdf - please download and complete the form and return to embopressproduction@wiley.com

EMBO Press participates in many Publish and Read agreements that allow authors to publish Open Access with reduced/no publication charges. Check your eligibility: https://authorservices.wiley.com/author-resources/Journal-Authors/open-access/affiliation-policies-payments/index.html

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2022-55472V3 and be addressed to emboreports@wiley.com.

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

EMBO Press Author Checklist

Corresponding Author Name: Rosa Puertollano
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2022-55472

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 2022)
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 replicates.
- 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 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.

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?	Yes	Materials and Methods

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	Yes	Appendix Table S4. Materials and Methods

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	Appendix Table S5. 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.	Yes	Appendix Table S4. Materials and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods

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.	Not Applicable	
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.	Yes	Materials and Methods

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	Acknowledgments 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	Based on our calculations we performed experiments in triplicate or more. For statistics, the number of cells counted is reflected for each experiment. A description of the statistical analysis methods can be found in Materials and Methods section
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	No blinding was done
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	No samples were excluded
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	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	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

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.	Not Applicable	
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.	Yes	Materials and Methods
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 approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
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?	Yes	Data Availability Section
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	