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ECM dimensionality tunes actin tension to modulate endoplasmic reticulum function and spheroid phenotypes of mammary epithelial cells

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Transaction Report:

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

1st Editorial Decision **28th Sep 2021**

Dear Dr Weaver,

Thank you again for the submission of your manuscript (EMBOJ-2021-109205) to The EMBO Journal and in addition providing us with a preliminary revision plan. As mentioned earlier, your study has been sent to three referees, and we have received reports from all of them, which I enclose below.

As you will see from their comments, the referees acknowledge the extensive analysis and potential interest and value of your findings linking actin cortex tension to ER response and MAMs in 2D versus 3D-cultured breast cancer cells, although they also express major concerns. In more detail, the referees state substantial issues regarding the depth and conceptual insight provided by the analyses, including on differential regulation of filamin in 3D conditions, and mechanistic detail explaining linkage of stiffness to ER signaling (ref#3, standfirst, pts.2,3; ref#2). Referee #2 is also concerned about subtlety of findings. In addition, there are major concerns regarding robustness of the findings and the technical setup used e.g. for 2D-3D measurements (ref#3, pt.1), statistics, data annotation as well as the image quality provided (ref#1, ref2; ref3, pt.2). Finally, the experts state that the high density of data and diversity of methods applied make the study difficult to follow, and they request major expansion of the respective annotation.

Given the interest stated and broader relevance of your findings for the field, we are overall able to invite you to revise your manuscript experimentally to address the referees' comments, along the lines sketched in your outline.

Please note that besides the technical robustness, i.p. the issues raised on mechanistic detail explaining the connections between 3D state-elicited tension, ECM ligation, filamin activity and ER-mitochondrial tethering/Ca2+ flux are important aspects for us to be consolidated and strengthened in the revised manuscript.

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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

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

Thank you for the opportunity to consider your work for publication.

I look forward to your revision.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

Instruction for the preparation of your revised manuscript:

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

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

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

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is

also reflected in the manuscript. The completed author checklist will also be part of the RPF.

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

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

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

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

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

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

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

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

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

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

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

Revision to The EMBO Journal should be submitted online within 90 days, unless an extension has been requested and approved by the editor; please click on the link below to submit the revision online before 27th Dec 2021:

Link Not Available

Referee #1:

The manuscript by Kai and colleagues addresses how extracellular matrix modifies plasma membrane tension and the consequences of this to the function of the endoplasmic reticulum.
The authors use a wide array of methods, and take advantage of transcriptome datasets to guide their studies. However, the analysis of these datasets is often subpar, and appears to show somewhat what the authors were hoping to see. Showing individual genes rather than just obscure GO categories would be more informative. The imaging throughout the manuscript is also not ideal, the images are small and blurred, making it hard to discern specific aspects. Despite the technical "flaws", the fact that so many parallel approaches were used does give confidence on the data.
My biggest concern is that the manuscript is extremely dense and very hard to read (and I am used to read papers with omics approaches...).
Regarding the novelty of the outcome, while it is reasonably shown that the extracellular matrix affects the tension of the plasma membrane, this paper brings a novel angle that the tension of the plasma membrane affects ER function. One can always wish that there would be further mechanistic details (and what happens to other organelles that are in close contact with the ER...), but a project is a project, and a message is a message.

Referee #2:

The manuscript by Kai and coworkers compare ER properties (morphology and functions) of single cells cultured in 2D and 3D conditions. To this end, the authors used two different MEC lines (MCF10A and HMT-3522 S-1) and different culture conditions: (1) 2D monolayer seeded on rBM-coated glass coverslips versus 3D organoids embedded in Matrigel; (2) cells seeded on compliant (74 Pa) PA gels coated with laminin-111 or Matrigel and cultured with either medium (2D) or laminin-111 or Matrigel supplemented medium (3D); (3) cells seeded on micropatterns coated with laminin-111 and cultured with either medium (2D) or laminin-111 supplemented medium (3D). Using these setups, the authors found that 2D- and 3D-cultured MECs similarly assemble F-actin at the cortex, however, differently distribute the Golgi. The authors show with these setups that in contrast to 2D-cultured MECs, 3D-cultured MECs downregulate filamin levels, diffusely distribute filamin in the cytoplasm, decrease filamin-dependent cortical actin tension, and display more efficient secretory protein trafficking, less ER stress, fewer ER-PM contacts, longer and more stable membrane protrusions and more vesicles in the sub-plasma membrane region. The link between filamin, cortical actin tension and ER structure and function was deciphered with shRNAs, chemical inhibitors and overexpression of conditionally-active ROCK and a battery of different methods ranging from RNAomics, SILAC, BioID, CLEM, cryoET, AFM, laser traps, TFM, etc.

The experimental models, experiments and the presentation of the data do not convince. It is questionable whether the 3D-single cell model faithfully resembles MECs in organoids or in the in vivo situation. A substantial characterization of these cell culture models (is laminin assembled around these cells, are integrins engaged in this assembly or are the different filamin levels in 2D/3D MECs affecting integrin-laminin ligation, how are polarity complexes distributed, does the size of the circular micropattern affect survival and/or proliferation, etc.) is essential to use them as surrogate for organoids and in vivo settings.
Although the methods used by the authors are impressive, results are not followed up (e.g. mechanism underlying differential filamin, SEC61, etc. expression; mechanism underlying differential regulation of cortical actin tension - Fig. 3a shows filamin puncta in F-actin-negative bleb-like structures in 2D cells; potential filamin association with integrins; mechanism underlying impaired SOCE, etc.), poorly controlled (are the ectopic expression levels of VSVGts405, ROCK, GFP-MAPPER, etc. comparable between 2D and 3D cultured MECs - e.g. if yes, why does for example, VSVGts405-GFP not accumulate inside 2D cells cultured at 32oC?), not always statistically evaluated (e.g. Fig. 2e, Fig. 4d, etc.), and often with very marginal differences between 2D and 3D (e.g. pEIF2a levels throughout the paper).
The presentation of experiments and their results needs improvement: heat maps are not labeled, Tables with differentially expressed genes and proteins are not available, seeding times of cells is rarely indicated, EM images are insufficiently described (how is an empty vesicle defined?) and of poor quality, material is not described (what is recombinant (r)BM and what is the difference between rBM and Matrigel?), cell lines are not indicated (are MCF10A and/or HMT-3522 S-1 displayed in figures?), etc.

In summary, this paper raises substantial concerns that require significant attention.

Referee #3:

In the current manuscript, Kai et al. establish an interesting relationship between the dimensionality of extracellular matrix and ER function of mammary epithelial cells (MCF10A). To directly compare the effect of matrix dimensionality while excluding other possible factors such as cell shape and multicellularity, the authors plated cells either on circular micropatterns of recombinant basement membrane (rBM), which prevented the cells from spreading thus maintaining a rounded shape (2D condition), or, for a 3D environment, overlaid similarly plated cells with an ECM matrix. Both conditions yielded individually plated cells, although

proliferation might over time lead to multiple cells on micropattern. That cell behavior can be vastly different depending on whether cells adhere to a 2D surface or are embedded in a 3D matrix is well documented in the literature. Previous studies fund that in 3D, mammary epithelial cells (MECs) become growth arrested, apoptosis-resistant, and they secret and assemble a basement membrane. In the current study, microarray analysis confirmed changes in expression of genes related to protein synthesis/secretion for cells in 3D and this matrix dimensionality-induced "ER phenotype" is further investigated. The authors use a wide range of techniques, including microarray expression analysis, RNAseq, proximity-based proteomics, SILAC, cryoET, AFM, microrheology, TFM, computational modeling, besides standard cell biology techniques.
A well-substantiated and original finding is a relationship between actin cortical stiffness and ER stress signaling, through differential expression of the crosslinker filamin. How filamin expression is tuned between 2D and 3D conditions and whether filamin is the only differentially-regulated protein important for affecting cortical stiffness remains unknown. Furthermore, how increased cortical stiffness induces ER stress remains at a rather speculative level, although the authors provide two possible models. The first is based on an observed increase of tubular plasma membrane protrusions when cortical tension is lower in 3D, and a postulated correlated increase in vesicle secretion. The other is based on altered ER-Ca2+ homeostasis, for which no direct evidence is provided besides a change in the number of ER-PM contact sites and slightly altered cytosolic Ca2+ dynamics. As discussed below, these models are not yet well-supported by data and not necessarily plausible.
Overall, the manuscript uses an original approach and offers an interesting new finding. However, there are technical and conceptual issues that will require substantial revisions. Moreover, what constitutes the molecular link between matrix dimensionality, differential filamin expression, and changes in ER function remains speculative. I therefore cannot recommend this manuscript for publication.

Major points:

1. Assessing differences between cells in 2D vs 3D
Several conclusions rely on differences assessed in light microscopy images between cells in 2D and in 3D. These include ER function based on ER morphology (Fig. 1l), tsO45-VSVG trafficking (2b-c), Ca2+ levels and dynamics (2d-e), ER-PM junctions (2i-j, 3c), Filamin localization (3a), Exo70-GFP localization (Fig. 6c), AnnexinA2 localization (Fig 6j). Although cells both in 2D and 3D contexts are rounded due to the ECM micropatterns, the fact that in 3D cells are surrounded by ECM could lead to the appearance of differential morphological/localization phenotypes that are not due to functional differences between the conditions. For example, cells in 2D might have a thicker actin cytoskeleton near the attachment surface, whereas adhesion-associated actin could be distributed more evenly around the entire cell circumference in the 3D assay. Cells in 2D could form ER-PM junctions preferentially near the adhesion surface in 2D, whereas ER-PM junctions are more uniformly distributed in 3D. Depending on how the images were quantified, from single-confocal slices, from maximum z-projections, or 3D reconstructions, the outcomes could be different. To address this, more information is needed to validate the quantifications.
Of particular concern are the Ca2+ measurements using FURA, and the VSVG trafficking assay. The Ca2+ measurements show a small difference between 2D and 3D, but the presence of a 3D matrix itself could change the ratiometric analysis due to distinct background (autofluorescence) between 2D and 3D. Even if this is not the case, are the differences observed biologically significant and the reason for differential ER function as implied? There are several ways for how concerns about the measurements themselves could be addressed- by calibrating the FURA measurements in both 2D and 3D, by comparing Ca2+ measurements in 3D between control cells and cells treated to have increased cortical tension, and/or by comparing Ca2+ measurements in 2D between control cells and cells with reduced cortical tension.
Regarding the VSVG trafficking assay, where plasma membrane/total VSVG-GFP signal was used to score secretion efficiency, it is unclear what was taken as total and what was taken as membrane. The final step of VSVG trafficking is from the Golgi to the PM. Fig. 1g shows that the Golgi is positioned at the top of cells in 2D and randomly in cells in 3D. The outcome of membrane/total measurements will strongly depend on what images were used for quantifications, e.g. individual confocal slices, MIPs, 3D reconstructions, and this is not specified.

2. Role of Ca2+.
In the discussion it is stated that "Given that we did not see an increase of intracellular calcium levels or SOCE under high actin tension (2D; high filamin expression), we speculate the ER-PM contact formation in cells ligated to an ECM in 2D serves as a feedback mechanism important for the re-establishment of calcium homeostasis". Fig. 2D in fact shows a statistically significant decrease of cytosolic Ca2+ in 2D relative to 3D, and a decrease in SOCE and on p8 this is interpreted as "[cells in 2D] not only had lower resting intracellular calcium content (Fig. 2d) but also exhibited compromised calcium regulation, as shown by a truncated amplitude of intracellular calcium release following thapsigargin treatment, and store-operated calcium entry (SOCE) (Fig. 2e)." A plausible explanation for these observations should be provided, which includes observations of changes in ER-PM contact sites, that clarify the observed phenotypes.

3. Cortical tension and secretion
Based on data in Fig. 5-6, the authors argue that increased cortical tension, such as in cells in 2D, changes plasma membrane dynamics and plasma membrane protein composition, which together reduce the ability of secretory vesicles to fuse and release their cargo. Furthermore, it is stated that vesicles accumulating near the PM in turn lead to ER stress. The data supporting this are not convincing to me. First, there is no evidence provided that vesicles backing up lead to ER stress and I am not aware of a precedent in the literature. Second, the data presented to demonstrate accumulation of secretory vesicles near the plasma membrane are inconclusive. Avoiding calling them "vesicles", the authors describe features in EM micrographs in Fig. 6e as "membrane-enclosed compartments", but implying they are vesicles. It is unclear how the structures were identified as secretory

vesicles and how it was excluded that they are folded plasma membrane protrusions, ER tubules, endosomal organelles etc. Is their size consistent with the size of secretory vesicles? How was it scored whether membrane-enclosed structures are filled with or "emptier" of macromolecular content? This also raises the question what the initial events are, following ECM ligation in 2D and 3D, leading to changes in cortical stiffness, ER stress, and secretion. How can ECM ligation in 2D/3D cause increased/decreased expression of filamin? These questions should be addressed at least in the discussion, if not experimentally.

4. Membrane protrusions and secretion
Based on Data in Fig. 5-6, the authors conclude that increased membrane protrusions facilitate vesicle secretion. This is based on computational modeling and the observation that decreased cortical tension leads to increased tubular membrane extensions, to which Exo70-GFP localizes. No direct evidence is provided showing that vesicle secretion preferentially occurs at the sites of membrane protrusions as opposed to flatter regions of the cell. To support the claim of membrane protrusions supporting vesicle secretion, a more direct demonstration is needed. Since cortical actin is well known to act as a barrier for vesicle secretion, how can it be excluded that increased secretion in cells in 3D environments is not primarily due to the decreased cortical stiffness, which also results in increased membrane protrusions?

5. Documentation and clarity
The large number of techniques used makes the manuscript sometimes difficult to follow, as little space is devoted to explaining the experimental conditions and analysis. It does not help that data mentioned in the main text is missing from the figures or that techniques are mentioned in the text for which not data is shown (see below). No supplemental data files are provided for the expression or proteomics analyses. The APEX analysis is mentioned to have yielded 13 differentially localized proteins, however, the authors should provide their identities of those proteins rather than categorizing them into 5 GO terms.

Minor points

1. P4, Fig. 1c. It is stated that spheroids in 3D acquire apical/basal polarity (presumably in contrast to 2D monolayers?), based E-cadherin, apical-lateral ZO1 and basal-lateral beta-4 integrin localization. However, only Laminin and beta4-intergrin staining are shown, not apical-lateral ZO1 or E-cadherin. The data provided neither demonstrate apical/basal polarity in 3D nor contrasting apical/basal polarity in 3D vs 2D. In addition to ZO1, E-cadherin, collagen IV, whey acidic protein and beta-casein are mentioned in the text, but no data is shown in the figures.
2. A paragraph in the methods section is devoted to correlative light and electron microscopy (CLEM) and data is mentioned in the text, but no CLEM data are shown.
3. GO analysis of 2D vs 3D: Were the four categories shown (SRP-dependent targeting to ER, Secretory lumen, Ca2+ homeostasis, ER UPR) the most significantly enriched GO terms among the differentially regulated genes?
4. Fig. 1l: concluding from low-res, saturated-appearing images of ER morphology towards ER function does not seem justified. I am not aware how ER function is reflected by the proximity of the ER to the cortex.
5. Images shown in Fig. 6c and 6j are of poor quality.
6. Fig. 2e - are the Ca2+ responses for the two conditions statistically significant? The responses should be shown as mean +/- S.D. and the difference be assessed by a suitable statistical test.
7. Filamin knockdown/overexpression. The efficiency of the chosen shRNA to knockdown filamin should be demonstrated by Western Blot or qPCR. Fig. 3G, 4A: What level of overexpression is achieved by the DOX-induced expression of FLMN? How do the artificially decreased/increased levels of filamin relate to the observed expression changes observed between cells plated in 2D and 3D?
8. Several constructs listed in the methods section were not used in experiments shown in this manuscript.
9. Figure legends should include what "n" means (number of cells, independent repeats, etc.).
10. Catalog numbers should be added for all commercially available reagents.

Rebuttal (Manuscript #EMBOJ-2021-109205)

We thank the reviewers for their constructive comments. The revised the manuscript addresses the reviewer's suggestions. The updated article includes mechanistic data linking cortical actin tension to endoplasmic reticulum (ER) stress and secretory regulation. The resubmitted manuscript incorporates several new figure panels that have been incorporated into the main and supplemental figures. The manuscript text has been edited and reflects the new results and address reviewer's comments. The revised manuscript includes an updated abstract, introduction and discussion that reflect the new mechanistic experimental findings and text edits that ensure the conclusions appropriately reflect the experimental data. Below is a summary of the mechanistic framework upon which our revised manuscript is based. We include a point-by-point rebuttal to each point raised by each of the reviewers. We are confident that the reviewers will agree that our manuscript is now suitable for publication at EMBO J.

Summary of new mechanistic findings: The updated manuscript includes new data demonstrating differential ECM ligation of an epithelial cell (two dimensions versus three dimensions; 2D versus 3D) modulates cellular phenotype by tuning cortical actin tension to regulate ER function. A key link between cortical actin tension and ER function is the actin cross linker filamin. Filamin is an actin-binding protein that has 24 immunoglobulin (Ig)-like repeats. The Ig20-21 domain of filamin is auto-inhibited under low mechanical force (Gehler, Baldassarre et al., 2009). High cortical actin tension (shown in the current article as occurring when an epithelial cell ligates its ECM in 2D) induces a conformational change in filamin that exposes its cryptic binding sites. The mechanosensitive region within the filamin molecule are within the same domain that binds to ER-resident stress-sensing proteins such as PERK and IRE1 (Urra, Henriquez et al., 2018, van Vliet, Giordano et al., 2017). Thus, under high cortical actin tension (2D ECM ligation) the mechanosensitive Ig21 domain of filamin unfolds and this region is now able to bind to both to the ER and to stress-sensing molecules including PERK. The result is an increase in cellular ER stress that compromises cell viability and protein secretion. Our revised manuscript provides causal evidence for this paradigm and includes substantial new experimental data presented in updated main and supplemental figures.

Referee #1:

The manuscript by Kai and colleagues addresses how extracellular matrix modifies plasma membrane tension and the consequences of this to the function of the endoplasmic reticulum. The authors use a wide array of methods, and take advantage of transcriptome datasets to guide their studies. However, the analysis of these datasets is often subpar, and appears to show somewhat what the authors were hoping to see. Showing individual genes rather than just obscure GO categories would be more informative. The imaging throughout the manuscript is also not ideal, the images are small and blurred, making it hard to discern specific aspects. Despite the technical "flaws", the fact that so many parallel approaches were used does give confidence on the data. My biggest concern is that the manuscript is extremely dense and very hard to read (and I am used to read papers with omics approaches...). Regarding the novelty of the outcome, while it is reasonably shown that the extracellular matrix affects the tension of the plasma membrane, this paper brings a novel angle that the tension of the plasma membrane affects ER function. One can always wish that there would be further mechanistic details (and what happens to other organelles that are in close contact with the ER...), but a project is a project, and a message is a message.

(1) The image contrast and resolution were improved using used Huygens Deconvolution Software.
(2) GO categories have been included and summarized in excel tables. A gene list for the RNAseq and microarray analyses has been included (see Table EV1-5).
(3) The manuscript text and presentation have been revised to improve clarity.

Referee #2:

The manuscript by Kai and coworkers compare ER properties (morphology and functions) of single cells cultured in 2D and 3D conditions. To this end, the authors used two different MEC lines (MCF10A and HMT-3522 S-1) and different culture conditions: (1) 2D monolayer seeded on rBM-coated glass coverslips versus 3D organoids embedded in Matrigel; (2) cells seeded on compliant (74 Pa) PA gels coated with laminin-111 or Matrigel and cultured with either medium (2D) or laminin-111 or Matrigel supplemented medium (3D); (3)

cells seeded on micropatterns coated with laminin-111 and cultured with either medium (2D) or laminin-111 supplemented medium (3D). Using these setups, the authors found that 2D- and 3D-cultured MECs similarly assemble F-actin at the cortex, however, differently distribute the Golgi. The authors show with these setups that in contrast to 2D-cultured MECs, 3D-cultured MECs downregulate filamin levels, diffusely distribute filamin in the cytoplasm, decrease filamin-dependent cortical actin tension, and display more efficient secretory protein trafficking, less ER stress, fewer ER-PM contacts, longer and more stable membrane protrusions and more vesicles in the sub-plasma membrane region. The link between filamin, cortical actin tension and ER structure and function was deciphered with shRNAs, chemical inhibitors and overexpression of conditionally-active ROCK and a battery of different methods ranging from RNAomics, SILAC, BioID, CLEM, cryoET, AFM, laser traps, TFM, etc.

The experimental models, experiments and the presentation of the data do not convince. It is questionable whether the 3D-single cell model faithfully resembles MECs in organoids or in the in vivo situation. A substantial characterization of these cell culture models (is laminin assembled around these cells, are integrins engaged in this assembly or are the different filamin levels in 2D/3D MECs affecting integrin-laminin ligation, how are polarity complexes distributed, does the size of the circular micropattern affect survival and/or proliferation, etc.) is essential to use them as surrogate for organoids and in vivo settings.

We respectfully point out that the intent of the studies summarized in our article was not to suggest that clarifying differences in the phenotype of single cells interacting with an ECM in 2D versus 3D will fully explain the complex physiology of patient derived organoids or 3D spheroids, nor of in vivo tissues. The studies summarized in the current article were designed to clarify one aspect of organoid/spheroid biology. We recognize that organoid morphogenesis includes self-organization mediated through cell-ECM and cell-cell adhesions and the acquisition of apico-basolateral polarity through complex cell biological mechanisms.

Although 3D organoid culture faithfully recapitulates some aspects of tissue-specific organ phenotype, it remains unclear how and why culturing epithelial cells in 2D induces such a fundamentally distinct phenotype from that induced in the same cells when they are ligating the same ECM but grown as a 2D monolayer. Indeed, cells plated on top of an ECM form well elaborated cell-cell junctions, generate apical-basal polarity, secrete apically, and growth arrest due to contact inhibition. Nevertheless, accumulating evidence indicate that the acquisition of apical-basal polarity achieved by cells plated as monolayers versus those plated within a 3D ECM is achieved differently (O'Brien, Zegers et al., 2002, Roignot, Peng et al., 2013, Yu, Datta et al., 2005). Furthermore, cells grown as 2D monolayers fail to demonstrate the same level of differentiation as illustrated by protein expression (Barcellos-Hoff, Aggeler et al., 1989, Li, Aggeler et al., 1987, Medina, Li et al., 1987, Roskelley, Desprez et al., 1994) and cells grown as 3D spheroids acquire long-term viability and sustained resistance to chemotherapy agents as compared to the same cells plated as 2D monolayers that exhibit increased apoptosis sensitivity (Boudreau, Werb et al., 1996, Weaver, Lelievre et al., 2002). While some of the distinct phenotypes exhibited by cells grown as organoids/spheroids has been deciphered much still remains to be determined.

In the current article we chose to ask a fundamental question. Do single cells interacting with an ECM in 2D versus 3D exhibit distinct phenotypes or are the differences induced by differential ECM ligation ONLY manifest at the multicellular level? To ensure that any differences we observed were not due to differences in the stiffness of the ECM the cells were engaging we used polyacrylamide gels with a stiffness “tuned” to recapitulate that measured in rBM hydrogels. To ensure that any differences we observed were not due to altered cell spreading phenotypes, we also used micropatterned substrates that provided equivalent ECM ligation but prevented cell spreading. Clearly, our findings indicate that even at the single cell level non-spread cells differentially express a large number of genes linked to ER stress and protein secretion and they also exhibit distinct phenotypes when they ligate their ECMs in 2D versus 3D. We then asked what was the molecular basis for this difference in the single cells ligating the ECM in 2D versus 3D? Our studies delineated a clear molecular mechanism that explains at least part of this differential phenotype. Importantly, we were the able to demonstrate that our studies at the single cell level can explain at least some of the phenotype of multi

cellular epithelial spheroids interacting with a rBM in 3D; including protein secretion and stress regulation. In conclusion, we contend that understanding how a fundamental difference in cellular phenotype induced by differential ligation of an ECM in 2D versus 3D can translate into a deeper understanding of the differential phenotype between monolayers of epithelial cells and organoid/spheroids with respect to not only gene expression but also differentiated/viability behavior. We could only achieve this objective by systemically deconstructing the impact of a single epithelial cell ligating an ECM (in this case a reconstituted basement membrane) in 2D versus 3D on gene expression and cellular phenotype. We hope the reviewer will agree that we have achieved this objective and that our findings do shed light upon clarifying the behavior of epithelial cells within 3D spheroids/organoids.

Although the methods used by the authors are impressive, results are not followed up (e.g. mechanism underlying differential filamin, SEC61, etc. expression; mechanism underlying differential regulation of cortical actin tension - Fig. 3a shows filamin puncta in F-actin-negative bleb-like structures in 2D cells; potential filamin association with integrins; mechanism underlying impaired SOCE, etc.), poorly controlled (are the ectopic expression levels of VSVGts405, ROCK, GFP-MAPPER, etc. comparable between 2D and 3D cultured MECs - e.g. if yes, why does for example, VSVGts405-GFP not accumulate inside 2D cells cultured at 32oC?), not always statistically evaluated (e.g. Fig. 2e, Fig. 4d, etc.), and often with very marginal differences between 2D and 3D (e.g. pElF2a levels throughout the paper).

(1) New experimental findings are included in the revised manuscript that provide a mechanistic basis for the differential phenotype of the epithelial cells interacting with ECM in 2D versus 3D. The revised manuscript has also been reorganized and the methods have been expanded. New figures have been included and incorporated into the main and supplemental figures.
(2) The expression levels of all genes examined has been verified and controlled and statistics have been included for all panels. Although there are instances, as the reviewer points out, that marginal differences were observed such as with pEIFa, we respectfully point out the differences are statistically significant. Nevertheless, to appease this reviewer's concerns we have now also included additional experiments to "back up" our initial findings that provide additional proof of the validity of our findings. We additionally included validation studies for expression levels of the key constructs including Exo70-GFP and GFP-MAPPER expressed ectopically under 2D and 3D conditions to ensure that any comparisons made and differential observations were not due to differential protein expression (see Fig EV2 for GFP-MAPPER and Fig EV5B for Exo70-GFP).
(3) Error bars have now been included for all main experimental figure panels and all figures throughout the article with the exception of one subpanel comprised of additional backup controls that were done once (Fig 4D left).

The presentation of experiments and their results needs improvement: heat maps are not labeled, Tables with differentially expressed genes and proteins are not available, seeding times of cells is rarely indicated, EM images are insufficiently described (how is an empty vesicle defined?) and of poor quality, material is not described (what is recombinant (r)BM and what is the difference between rBM and Matrigel?), cell lines are not indicated (are MCF10A and/or HMT-3522 S-1 displayed in figures?), etc. In summary, this paper raises substantial concerns that require significant attention.

(1) Heat maps have now been regenerated and included in the revised article to improve clarity (see Fig 1A, 1I, EV1A and EV1B). Labels have also been included for all figures.
(2) Excel files with differentially expressed genes and proteins have been created (see Table EV1, Table EV2, Table EV3, Table EV4, and Table EV5), and we have included all listed experimental details in the figure legends. The Methods section has been expanded to include additional information as requested by the reviewer, including cell lines used and seeding times.
(3) A sentence has been included to define rBM as follows: "rBM resembles the laminin/collagen IV-rich basement membrane extracellular environment found in many tissues"
(4) All reference to Matrigel has been replaced with rBM.

(5) The revised manuscript includes the following paragraph within the results section to describe the distinct vesicle types:
"The assessment for filled versus empty was done by visual inspection of the three-dimensional cryo-ET reconstructions. Compartments that contained discernible, individual macromolecules were counted as "filled". Compartments with somewhat smoother but very dense material were also counted as "filled". All others were counted as "empty". The process was repeated independently three times to compile the statistics."

Referee #3:
In the current manuscript, Kai et al. establish an interesting relationship between the dimensionality of extracellular matrix and ER function of mammary epithelial cells (MCF10A). To directly compare the effect of matrix dimensionality while excluding other possible factors such as cell shape and multicellularity, the authors plated cells either on circular micropatterns of recombinant basement membrane (rBM), which prevented the cells from spreading thus maintaining a rounded shape (2D condition), or, for a 3D environment, overlaid similarly plated cells with an ECM matrix. Both conditions yielded individually plated cells, although proliferation might over time lead to multiple cells on micropattern. That cell behavior can be vastly different depending on whether cells adhere to a 2D surface or are embedded in a 3D matrix is well documented in the literature. Previous studies fund that in 3D, mammary epithelial cells (MECs) become growth arrested, apoptosis-resistant, and they secret and assemble a basement membrane. In the current study, microarray analysis confirmed changes in expression of genes related to protein synthesis/secretion for cells in 3D and this matrix dimensionality-induced "ER phenotype" is further investigated. The authors use a wide range of techniques, including microarray expression analysis, RNAseq, proximity-based proteomics, SILAC, cryoET, AFM, microrheology, TFM, computational modeling, besides standard cell biology techniques.

A well-substantiated and original finding is a relationship between actin cortical stiffness and ER stress signaling, through differential expression of the crosslinker filamin. How filamin expression is tuned between 2D and 3D conditions and whether filamin is the only differentially-regulated protein important for affecting cortical stiffness remains unknown. Furthermore, how increased cortical stiffness induces ER stress remains at a rather speculative level, although the authors provide two possible models. The first is based on an observed increase of tubular plasma membrane protrusions when cortical tension is lower in 3D, and a postulated correlated increase in vesicle secretion. The other is based on altered ER-Ca2+ homeostasis, for which no direct evidence is provided besides a change in the number of ER-PM contact sites and slightly altered cytosolic Ca2+ dynamics. As discussed below, these models are not yet well-supported by data and not necessarily plausible.

We include new data to demonstrate on how cortical actin tension can influence ER calcium homeostasis and ER stress via the actin cross linker filamin. Our data indicate that cortical actin tension can expose the mechanosensitive domain of filamin, as has been reported previously (Gehler, Baldassarre et al., 2009, Razinia, Makela et al., 2012). The mechanosensitive region of filamin overlaps with the filamin-PERK interaction site (Fig 5F). Thus, when cortical actin tension is high, filamin unfolding is favored and the molecule is competent to binding to the stress protein PERK. High filamin-PERK-ER linkage associates with elevated ER stress, reduced ER calcium and a compensatory increase in ER-PM membrane contact sites. The net result is an increase in cellular ER stress and compromised protein secretion. We were able to ameliorate this ER "stressed" phenotype (ER calcium content, pEIF2a level, and ER-PM membrane contact formation) by reducing actin tension using blebbistatin and by overexpressing the dominant negative filamin mechanosensitive domain immunoglobulin domains Ig21-23 (which also inhibit filamin-PERK interactions). We contend that these studies are the first to show that ER is a mechanosensitive organelle. Our findings are consistent with recent studies demonstrating that mechanically stretching of cells can activate ER calcium leakage (Nava, Miroshnikova et al., 2020).

Our revised manuscript includes new findings regarding the level/involvement of another actin binding protein alpha actinin (see Appendix Figure S1A). Consistent with prior studies implicating filamin and not alpha actinin

in mechanotransduction, we found no differences in the level of this actin binding protein in the epithelial cells interacting with ECM in 2D versus 3D. Knockdown of alpha actinin also had no discernible impact on cell viability phenotype (Fig 8B).

Overall, the manuscript uses an original approach and offers an interesting new finding. However, there are technical and conceptual issues that will require substantial revisions. Moreover, what constitutes the molecular link between matrix dimensionality, differential filamin expression, and changes in ER function remains speculative. I therefore cannot recommend this manuscript for publication.

The revised manuscript includes mechanistic studies linking cortical actin tension to ER function.

Major points:
1. Assessing differences between cells in 2D vs 3D
Several conclusions rely on differences assessed in light microscopy images between cells in 2D and in 3D. These include ER function based on ER morphology (Fig. 1l), tsO45-VSVG trafficking (2b-c), Ca2+ levels and dynamics (2d-e), ER-PM junctions (2i-j, 3c), Filamin localization (3a), Exo70-GFP localization (Fig. 6c), AnnexinA2 localization (Fig 6j). Although cells both in 2D and 3D contexts are rounded due to the ECM micropatterns, the fact that in 3D cells are surrounded by ECM could lead to the appearance of differential morphological/localization phenotypes that are not due to functional differences between the conditions. For example, cells in 2D might have a thicker actin cytoskeleton near the attachment surface, whereas adhesion-associated actin could be distributed more evenly around the entire cell circumference in the 3D assay. Cells in 2D could form ER-PM junctions preferentially near the adhesion surface in 2D, whereas ER-PM junctions are more uniformly distributed in 3D. Depending on how the images were quantified, from single-confocal slices, from maximum z-projections, or 3D reconstructions, the outcomes could be different. To address this, more information is needed to validate the quantifications.

To address the possibility that our studies failed to represent the phenotype of the imaged cells we repeated our cellular protein imaging distribution analysis. We imaged each cell for level/organization of the ER-PM reporter and the Exo70-GFP dividing our analysis into three regions (apical, middle, and basal region) (see Fig EV2 and EV5B). We did not document any polarized distribution of the ER-PM reporter protein or the Exo70-GFP in any of the cells analyzed. Instead, as reported in our first submission, we found that the differential distribution of ER-PM contact sites and Exo70-GFP were comparable between the apical, middle and bottom section of the cells.

Of particular concern are the Ca2+ measurements using FURA, and the VSVG trafficking assay. The Ca2+ measurements show a small difference between 2D and 3D, but the presence of a 3D matrix itself could change the ratiometric analysis due to distinct background (autofluorescence) between 2D and 3D. Even if this is not the case, are the differences observed biologically significant and the reason for differential ER function as implied? There are several ways for how concerns about the measurements themselves could be addressed- by calibrating the FURA measurements in both 2D and 3D, by comparing Ca2+ measurements in 3D between control cells and cells treated to have increased cortical tension, and/or by comparing Ca2+ measurements in 2D between control cells and cells with reduced cortical tension.

The steady state cytosolic calcium difference between 2D and 3D is small but statistically significant. Our revised manuscript now includes a differential analysis that includes a more robust method that quantifies dynamic ER Ca^{2+} levels using the FURA assay. Our new results quantified ER Ca^{2+} content (ΔF) by assessing the change in cytosolic Ca^{2+} level in response to Thapsigargin treatment (which triggers ER-Ca^{2+} content release). This approach is a more relevant measurement which assesses the impact of ligation of ECM in 2D versus 3D on ER homeostasis. The approach also addresses another reviewer concerns because fluorescent intensity is self-corrected within each sample and any relative difference measured in the cells interacting with the ECM in 2D versus 3D will not be influenced by the additional ECM ligation or ECM overlay. To further ensure that the changes we observed was not due to the presence of an additional layer of rBM we also directly

compared the ER Ca^{2+} level between control cells interacting with rBM in 2D and the same cells interacting with rBM in 2D that were treated with blebbistatin to reduce cortical actin tension (Fig 5A).

Regarding the VSVG trafficking assay, where plasma membrane/total VSVG-GFP signal was used to score secretion efficiency, it is unclear what was taken as total and what was taken as membrane. The final step of VSVG trafficking is from the Golgi to the PM. Fig. 1g shows that the Golgi is positioned at the top of cells in 2D and randomly in cells in 3D. The outcome of membrane/total measurements will strongly depend on what images were used for quantifications, e.g. individual confocal slices, MIPs, 3D reconstructions, and this is not specified.

(1) The methods section was expanded to include an explanation for how secretion efficiency was quantified. "Actin cortex (stained by phalloidin) was used as a proxy for the plasma membrane location. VSVG fluorescent signals overlapping with actin cortex were quantified as plasma membrane localized VSVG. The secretion efficiency was scored as VSVG-ts045-GFP fluorescence at the plasma membrane relative to total VSVG-ts045-GFP fluorescence for the entire cell."

(2) VSV-G-GFP is co-translationally inserted into the ER during its biogenesis pathway; thus, we contend that it is appropriate to normalize to the total cell fluorescent intensity to ensure that the amount of the protein observed at the plasma membrane is not influenced by the total cellular level of VSVG-GP expressed.

(3) We do not observe significant difference in Exo70-GFP protein at the apical, middle and basal section of cells interacting with an ECM in 2D versus 3D. VSVG-trafficking requires Exo70 docking at the plasma membrane (Liu, Zuo et al., 2007). Our Exo70-GFP quantification of apical, middle, basal section of the cells (Fig EV5B) clearly demonstrate that there is no differential localization of Exo70-GFP in the different regions of the cells. Thus, we do not expect that VSVG will be preferentially localized to different domains of cells.

2. Role of Ca2+.
In the discussion it is stated that "Given that we did not see an increase of intracellular calcium levels or SOCE under high actin tension (2D; high filamin expression), we speculate the ER-PM contact formation in cells ligated to an ECM in 2D serves as a feedback mechanism important for the re-establishment of calcium homeostasis". Fig. 2D in fact shows a statistically significant decrease of cytosolic Ca2+ in 2D relative to 3D, and a decrease in SOCE and on p8 this is interpreted as "[cells in 2D] not only had lower resting intracellular calcium content (Fig. 2d) but also exhibited compromised calcium regulation, as shown by a truncated amplitude of intracellular calcium release following thapsigargin treatment, and store-operated calcium entry (SOCE) (Fig. 2e)." A plausible explanation for these observations should be provided, which includes observations of changes in ER-PM contact sites, that clarify the observed phenotypes.

The following paragraph is now included in the discussion section to clarify this issue.

"SOCE is a key molecular regulator of calcium homeostasis in the cell. When ER calcium stores are depleted, the ER rapidly establishes ER-PM contact to restore calcium influx at the plasma membrane needed to replenish the cellular ER calcium repository. Surprisingly, we determined that the higher numbers of ER-PM contact sites in the cells interacting with the ECM in 2D (high actin tension) was not accompanied by increased levels of intracellular calcium (Fig 2D and 2E), likely because ER calcium stores are already depleted in these cells and this in turn compromises calcium homeostasis. Indeed, we observed lower levels of ER calcium storage when the cells were ligating the ECM in 2D. This observation is consistent that earlier work showing that mechanical stretch can activate ER calcium leakage into the cytoplasm. Thus, we interpret our findings to mean that the higher number of ER-PM sites is a compensatory cellular mechanism that is induced to restore calcium homeostasis to compensate for low levels of ER calcium."

3. Cortical tension and secretion
Based on data in Fig. 5-6, the authors argue that increased cortical tension, such as in cells in 2D, changes plasma membrane dynamics and plasma membrane protein composition, which together reduce the ability of

secretory vesicles to fuse and release their cargo. Furthermore, it is stated that vesicles accumulating near the PM in turn lead to ER stress. The data supporting this are not convincing to me. First, there is no evidence provided that vesicles backing up lead to ER stress and I am not aware of a precedent in the literature. Second, the data presented to demonstrate accumulation of secretory vesicles near the plasma membrane are inconclusive. Avoiding calling them "vesicles", the authors describe features in EM micrographs in Fig. 6e as "membrane-enclosed compartments", but implying they are vesicles. It is unclear how the structures were identified as secretory vesicles and how it was excluded that they are folded plasma membrane protrusions, ER tubules, endosomal organelles etc. Is their size consistent with the size of secretory vesicles? How was it scored whether membrane-enclosed structures are filled with or "emptier" of macromolecular content? This also raises the question what the initial events are, following ECM ligation in 2D and 3D, leading to changes in cortical stiffness, ER stress, and secretion. How can ECM ligation in 2D/3D cause increased/decreased expression of filamin? These questions should be addressed at least in the discussion, if not experimentally.

(1) Although there is no precedent in the literature that a decrease in secretion elicits ER stress, we maintain that it is reasonable to conclude that alterations in cellular trafficking traps proteins in the ER and that this would thereafter induce ER stress. We have included this possibility in our discussion section.

(2) In three-dimensional cryo-ET reconstructions, many types of membrane structures, for example ER, mitochondria, phagosomes, coated vesicles, are identifiable by their shape, content, and texture. Our analysis did not include anything that can be obviously excluded from being secretory vesicles. Folded plasma membrane protrusions are identifiable in most cases because the volume is three dimensional and it is thus possible to follow the path of the membrane in three dimensions. Thus, we agree that here may still be a mix of secretory vesicles and other types of membrane-enclosed compartments that are not readily identifiable. For these reasons we opted to use the term "membrane-enclosed compartment" instead of "secretory vesicles". Nevertheless, it is likely a majority of the imaged compartments correspond to secretory vesicles and that the trends we identified reflect secretory vesicle phenotype.

(3) A paragraph has been included in the result section to describe how the vesicles were categorized.

“The assessment for filled versus empty was done by visual inspection of the three-dimensional cryo-ET reconstructions. Compartments that contained discernible, individual macromolecules were counted as "filled". Compartments with somewhat smoother but very dense material were also counted as "filled". All others were counted as "empty". The process was repeated independently three times to compile the statistics.”

(3) The revised manuscript includes considerable new mechanistic data that clarifies how cortical actin tension tunes ER structure/function. See summary on page one and earlier rebuttal comments.

4. Membrane protrusions and secretion
Based on Data in Fig. 5-6, the authors conclude that increased membrane protrusions facilitate vesicle secretion. This is based on computational modeling and the observation that decreased cortical tension leads to increased tubular membrane extensions, to which Exo70-GFP localizes. No direct evidence is provided showing that vesicle secretion preferentially occurs at the sites of membrane protrusions as opposed to flatter regions of the cell. To support the claim of membrane protrusions supporting vesicle secretion, a more direct demonstration is needed. Since cortical actin is well known to act as a barrier for vesicle secretion, how can it be excluded that increased secretion in cells in 3D environments is not primarily due to the decreased cortical stiffness, which also results in increased membrane protrusions?

Our findings suggest actin tension could modulate membrane protrusions to facilitate vesicle secretion. We revised and tempered our conclusions and discussion to reflect these results.

5. Documentation and clarity
The large number of techniques used makes the manuscript sometimes difficult to follow, as little space is devoted to explaining the experimental conditions and analysis. It does not help that data mentioned in the main text is missing from the figures or that techniques are mentioned in the text for which not data is shown (see

below). No supplemental data files are provided for the expression or proteomics analyses. The APEX analysis is mentioned to have yielded 13 differentially localized proteins, however, the authors should provide their identities of those proteins rather than categorizing them into 5 GO terms.

(1) Excel spreadsheets of the proteomic analyses and significant genes enriched from the RNAseq experiments have now been included (see Table EV1, Table EV2, Table EV3, Table EV4, and Table EV5).

(2) GO terms have been replaced with protein identities in the main figure.

(3) Citations have now been included to describe prior work pertaining to the localization of E-cadherin in cellular monolayers versus spheroids.

Minor points

1. P4, Fig. 1c. It is stated that spheroids in 3D acquire apical/basal polarity (presumably in contrast to 2D monolayers?), based E-cadherin, apical-lateral ZO1 and basal-lateral beta-4 integrin localization. However, only Laminin and beta4-intergrin staining are shown, not apical-lateral ZO1 or E-cadherin. The data provided neither demonstrate apical/basal polarity in 3D nor contrasting apical/basal polarity in 3D vs 2D. In addition to ZO1, E-cadherin, collagen IV, whey acidic protein and beta-casein are mentioned in the text, but no data is shown in the figures.

The revised manuscript includes relevant citations and rationale for both the choice of markers and descriptors of cellular phenotype in monolayers versus spheroid culture.

2. A paragraph in the methods section is devoted to correlative light and electron microscopy (CLEM) and data is mentioned in the text, but no CLEM data are shown.

The CLEM and associated data and references have been removed.

3. GO analysis of 2D vs 3D: Were the four categories shown (SRP-dependent targeting to ER, Secretory lumen, Ca2+ homeostasis, ER UPR) the most significantly enriched GO terms among the differentially regulated genes?

The GO terms we included in our manuscript are not the most significantly enriched terms among the differentially regulated genes. We purposely chose to focus on the GO terms listed above to explore phenotype/genotype/dimensionality interactions. As indicated in the introduction, cells embedded within rBM (engaging an ECM in 3D) secrete endogenous basement membrane and are very stress-resilient. Given the endoplasmic reticulum is a key organelle involved in protein secretion and stress sensing of cells, we confined our GO term presentation to the more relevant pathways/GO terms that reflect the phenotype we were examining.

4. Fig. 1l: concluding from low-res, saturated-appearing images of ER morphology towards ER function does not seem justified. I am not aware how ER function is reflected by the proximity of the ER to the cortex.

The offending low resolution images have been removed. New images now have been included that present similar findings demonstrated using the GFP-MAPPER reporter (the fluorescent reporter that marks plasma membrane-proximal ER; see Fig. 2).

5. Images shown in Fig. 6c and 6j are of poor quality.

Huygens Deconvolution Software was used to improve the contrast and resolution the articles images.

6. Fig. 2e - are the Ca2+ responses for the two conditions statistically significant? The responses should be shown as mean +/- S.D. and the difference be assessed by a suitable statistical test.

S.D. has been included in our representative calcium assay. A student's t-test has been included for ΔF/F graph for the three independent experiments.

7. Filamin knockdown/overexpression. The efficiency of the chosen shRNA to knockdown filamin should be demonstrated by Western Blot or qPCR. Fig. 3G, 4A: What level of overexpression is achieved by the DOX-induced expression of FLMN? How do the artificially decreased/increased levels of filamin relate to the observed expression changes observed between cells plated in 2D and 3D?

Western blot information has been included to confirm filamin shRNA knockdown and to demonstrate filamin overexpression filamin relative to abundance of filamin level in cells engaging the ECM in 2D versus 3D (see Fig 3E and 3G).

8. Several constructs listed in the methods section were not used in experiments shown in this manuscript.

Superfluous construct descriptions have been deleted.

9. Figure legends should include what "n" means (number of cells, independent repeats, etc.).

Figure legends now include the requested information such as number of cells, independent repeats, "n" definition etc.

10. Catalog numbers should be added for all commercially available reagents.

All catalog numbers for commercial reagents have been included.

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Dear Dr Valerie Weaver,

Thank you for submitting your revised manuscript (EMBOJ-2021-109295R) to The EMBO Journal, as well as for your patience with our feedback, which got protracted by delayed reviewer input. Your amended study was sent back to the referees for their re-evaluation, and we have received comments from two of them, which I enclose below. As you will see, the experts stated that the work has been substantially improved by the complementary work and they are now in favour of publication, pending minor revision.

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

Please consider the remaining minor comments of the reviewers regarding conceptual integration of your findings and technicalities carefully and amend the text and discussion accordingly where appropriate.

Also, we now need you to take care of a number of minor issues related to formatting and data presentation as detailed below, which should be addressed at re-submission.

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

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

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

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

with
Best regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Please limit the keywords to the manuscript to maximally five.

>> Data availability section: remove the referee tokens from the data accessibility section and release privacy.

>> Reference format: please limit to10 authors before et al. .

>> Dataset EV Legends: Table EV1-6 should be renamed to 'Datasets EV1-6'; remove legends from the manuscript and add to corresponding files.

>> Appendix file: merge the current appendix figure with Table S1 and Figure S1 (which will need renaming) in one appendix PDF with a title page including a ToC, and Appdix Figure legends. Appendix Table S1 and appendix figure should be removed from M&M and callouts should be added.

>> Please adjust the title of the 'Declaration of Competing Interests' section to 'Disclosure and Competing Interests Statement'.

>> Enter the funding information including project numbers in our online system for consistency. Thank you!

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

details on the author's contribution. More information is available in our guide to authors.
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>> Please consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode. Recheck i.p. organization and annotation of Figure EV4.

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Use the link below to submit your revision:

Link Not Available

Referee #2:

(1) The quality of the paper improved significantly. The appeasement worked out.

(2) I do not have a problem with the fundamental question that the authors wanted to addressed in this paper, but rather whether the fundamental question is accurately answered. The differences between 2D and 3D single cells are indeed very interesting, however, I have doubts whether a simple overexpression of caROCK in organoids and a TRAIL treatment of 2D cells and organoids suffice to demonstrate that all the 3D versus 2D single cell findings can be readily transferred to an organoid. Is the contractility of a 2D cell cortex comparable to a caROCK expressing 3D cortex?

Referee #3:

"ECM dimensionality tunes actin tension to modulate the endoplasmic reticulum and
spheroid phenotype", revised manuscript by Kai et al., for consideration by EMBO Journal

In their revised manuscript, Kai et al. have addressed several of the concerns that I have raised in my assessment of the previous submission. In particular, they have added new data providing insight into the mechanistic link between Filamin, the actin cortex and the ER stress response, by showing a contractility-dependent interaction between PERK and Filamin, using a proximity ligation assay. Together, this has led to a much-improved manuscript, although some of my concerns have remained insufficiently addressed (see below).
What I find is still missing is a plausible model in which the authors describe a possible sequence of events for how matrix dimensionality leads to ER stress, consistent with all their data. They provide two possible mechanisms in the discussion (P24) - one through filamin-PERK interaction and impaired Ca2+ homeostasis, for which evidence is provided, the other through secretory vesicles "backing up", as a stiffer cortex is less likely to recruit the exocyst component Exo70 in support of vesicle secretion. For the latter, while significant space is devoted in the manuscript to explore this aspect (Fig. 6, 7) no evidence is provided for how secretory vesicles accumulating at the plasma membrane in turn could cause ER stress.

My previous comment:
"Regarding the VSVG trafficking assay, where plasma membrane/total VSVG-GFP signal was used to score secretion efficiency, it is unclear what was taken as total and what was taken as membrane. The final step of VSVG trafficking is from the Golgi to the PM. Fig. 1g shows that the Golgi is positioned at the top of cells in 2D and randomly in cells in 3D. The outcome of membrane/total measurements will strongly depend on what images were used for quantifications, e.g. individual confocal slices, MIPs, 3D reconstructions, and this is not specified."

My remaining concern:
The way the VSVG trafficking assay has been quantified still has not been explained. Were maximum intensity-projections quantified, confocal slices from the bottom/middle/top? Since the Golgi position changes between 2D and 3D, this could be important.

My previous comment:
"Based on data in Fig. 5-6, the authors argue that increased cortical tension, such as in cells in 2D, changes plasma membrane

dynamics and plasma membrane protein composition, which together reduce the ability of secretory vesicles to fuse and release their cargo. Furthermore, it is stated that vesicles accumulating near the PM in turn lead to ER stress. The data supporting this are not convincing to me. First, there is no evidence provided that vesicles backing up lead to ER stress and I am not aware of a precedent in the literature. Second, the data presented to demonstrate accumulation of secretory vesicles near the plasma membrane are inconclusive. Avoiding calling them "vesicles", the authors describe features in EM micrographs in Fig. 6e [Fig. 7E in the revised manuscript] as "membrane-enclosed compartments", but implying they are vesicles. It is unclear how the structures were identified as secretory vesicles and how it was excluded that they are folded plasma membrane protrusions, ER tubules, endosomal organelles etc. Is their size consistent with the size of secretory vesicles? How was it scored whether membrane-enclosed structures are filled with or "emptier" of macromolecular content? This also raises the question what the initial events are, following ECM ligation in 2D and 3D, leading to changes in cortical stiffness, ER stress, and secretion. How can ECM ligation in 2D/3D cause increased/decreased expression of filamin? These questions should be addressed at least in the discussion, if not experimentally."

My remaining concern:
In their response, the authors agreed that there is no precedent in the literature for secretory vesicle accumulation at the PM causing ER stress. Given that no further evidence supporting this has been provided, I still don't find this to be plausible model. The questions of whether the vesicles' sizes in the EM micrographs are consistent with secretory vesicles have not been addressed. With a scale bar of 200nm in Fig. 7e, some of the vesicles are 1µm in size and larger. In the micrographs shown in Fig. 7E, adjacent to the "membrane-enclosed compartments", there appears to be another membrane, which would make it unlikely that the compartments are intracellular. Given that cryo-ET data are available, more convincing data should be presented or the data removed. It furthermore remains unclear how filled/empty vesicles were determined. The methods section specifies that "filled" compartments were those with "discernible, individual macromolecules" (which is unlikely to be possible) or those with "somewhat smoother but very dense material". The documentation for this needs to be improved, as the current description appears to be subjective. For Fig. 7F, a p-value is given, but no error bars are shown, it is unclear how many cells/fields from how many independent experiments the bar graph in 7F was derived from. The y-axis unit is given as "number of vesicles" - per cell, per field of view, per tomogram, per unit length of plasma membrane?

My previous comment:
"2. A paragraph in the methods section is devoted to correlative light and electron microscopy (CLEM) and data is mentioned in the text, but no CLEM data are shown."

My remaining concern:
Although stated otherwise in the rebuttal, CLEM analysis is still mentioned in main text, methods, and acknowledgements, but no CLEM data are shown.

Minor:
Fig. 5G: singular "punctum" plural "puncta" (Y-axis label). The y-axis unit is given as puncta per cell (x10^3), but it appears to be a relative measurement to the untreated 3D condition, which is set to 1, since there don't seem to be 1000 puncta in the images shown.

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We thank the reviewers for their constructive comments. We have updated the text in the manuscript according to their suggestions.

Referee #2:

(1) The quality of the paper improved significantly. The appeasement worked out.

(2) I do not have a problem with the fundamental question that the authors wanted to addressed in this paper, but rather whether the fundamental question is accurately answered. The differences between 2D and 3D single cells are indeed very interesting, however, I have doubts whether a simple overexpression of caROCK in organoids and a TRAIL treatment of 2D cells and organoids suffice to demonstrate that all the 3D versus 2D single cell findings can be readily transferred to an organoid. Is the contractility of a 2D cell cortex comparable to a caROCK expressing 3D cortex?

We agree with the reviewer that simple overexpression of ROCK experiment will not comprehensively demonstrate that our 2D and 3D single cell findings are readily transferred to the spheroid context. Nevertheless, we contend that this key experiment does verify that our findings using the single cell system that actin tension differentially influences ER stress in cells in 2D versus 3D can be translated to the spheroid context where we quantified increased tension-induced stress and chemotherapy sensitivity.

As shown in Figure 4A, our AFM measurement demonstrated that the actin tension quantified in cells ligating to the ECM in two dimension and that of ROCK-expressing cells ligating to the ECM in three dimensions are very similar (not significantly different, $p = 0.2887$). Given that actin tension is regulated by actomyosin contractility, we maintain that our conclusion that the contractility of the cortical actin cortex in the cells ligating the ECM in 2D is comparable to that measured in the ROCK-expressing cells ligating the ECM in 3D.

Referee #3:

"ECM dimensionality tunes actin tension to modulate the endoplasmic reticulum and spheroid phenotype", revised manuscript by Kai et al., for consideration by EMBO Journal

In their revised manuscript, Kai et al. have addressed several of the concerns that I have raised in my assessment of the previous submission. In particular, they have added new data providing insight into the mechanistic link between Filamin, the actin cortex and the ER stress response, by showing a contractility-dependent interaction between PERK and Filamin, using a proximity ligation assay. Together, this has led to a much-improved manuscript, although some of my concerns have remained insufficiently addressed (see below).

What I find is still missing is a plausible model in which the authors describe a possible sequence of events for how matrix dimensionality leads to ER stress, consistent with all their data. They provide two possible mechanisms in the discussion (P24) - one through filamin-PERK interaction and impaired Ca2+ homeostasis, for which evidence is provided, the other through

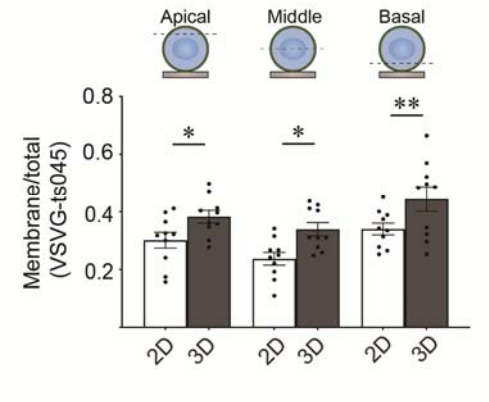
secretory vesicles "backing up", as a stiffer cortex is less likely to recruit the exocyst component Exo70 in support of vesicle secretion. For the latter, while significant space is devoted in the manuscript to explore this aspect (Fig. 6, 7) no evidence is provided for how secretory vesicles accumulating at the plasma membrane in turn could cause ER stress.

Despite the fact that we did not present evidence that the accumulation of secretory vesicles at the plasma membrane causes ER stress, we maintain that this possibility is highly likely. Moreover, there are examples in the literature that inhibiting protein secretion induces ER stress. Accordingly, we intend to retain these data presented in Figures 6 and 7.

My previous comment:
"Regarding the VSVG trafficking assay, where plasma membrane/total VSVG-GFP signal was used to score secretion efficiency, it is unclear what was taken as total and what was taken as membrane. The final step of VSVG trafficking is from the Golgi to the PM. Fig. 1g shows that the Golgi is positioned at the top of cells in 2D and randomly in cells in 3D. The outcome of membrane/total measurements will strongly depend on what images were used for quantifications, e.g. individual confocal slices, MIPs, 3D reconstructions, and this is not specified."

My remaining concern:
The way the VSVG trafficking assay has been quantified still has not been explained. Were maximum intensity-projections quantified, confocal slices from the bottom/middle/top? Since the Golgi position changes between 2D and 3D, this could be important.

In our revised manuscript, we now include a paragraph describing how we quantified the VSV-trafficking assay in M&M section (Under Image Quantification subsection). We did not quantify VSVG at different focal planes in our first revision because VSVG-trafficking requires Exo70 docking at the plasma membrane (Liu, Zuo et al., 2007) and our Exo70-GFP quantification of apical, middle, basal section of the cells (Fig EV5B) clearly demonstrated that there was no differential localization of Exo70-GFP in the different regions of the cells. Accordingly, we do not expect that VSVG will be preferentially localized to different domains of cells. However, to ensure that our assumptions and data analysis are indeed accurate, and to address the reviewers concern we rigorously repeated the experiment/analysis. Consistent with our earlier prediction, once again we found that cells ligating the ECM in 3D have higher VSVG-GFP levels at all focal points (see below; n=10 cells per condition from one experiment).



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My previous comment:
"Based on data in Fig. 5-6, the authors argue that increased cortical tension, such as in cells in 2D, changes plasma membrane dynamics and plasma membrane protein composition, which together reduce the ability of secretory vesicles to fuse and release their cargo. Furthermore, it is stated that vesicles accumulating near the PM in turn lead to ER stress. The data supporting this are not convincing to me. First, there is no evidence provided that vesicles backing up lead to ER stress and I am not aware of a precedent in the literature. Second, the data presented to demonstrate accumulation of secretory vesicles near the plasma membrane are inconclusive. Avoiding calling them "vesicles", the authors describe features in EM micrographs in Fig. 6e [Fig. 7E in the revised manuscript] as "membrane-enclosed compartments", but implying they are vesicles. It is unclear how the structures were identified as secretory vesicles and how it was excluded that they are folded plasma membrane protrusions, ER tubules, endosomal organelles etc. Is their size consistent with the size of secretory vesicles? How was it scored whether membrane-enclosed structures are filled with or "emptier" of macromolecular content? This also raises the question what the initial events are, following ECM ligation in 2D and 3D, leading to changes in cortical stiffness, ER stress, and secretion. How can ECM ligation in 2D/3D cause increased/decreased expression of filamin? These questions should be addressed at least in the discussion, if not experimentally."

My remaining concern:
In their response, the authors agreed that there is no precedent in the literature for secretory vesicle accumulation at the PM causing ER stress. Given that no further evidence supporting this has been provided, I still don't find this to be plausible model.

Although we do not present evidence to demonstrate that accumulation of secretory vesicles at the plasma membrane causes ER stress, based upon prior published evidence that inhibiting protein secretion induces ER stress, we are confident this assumption is accurate. Accordingly, we have opted to retain these data in Figures 6 and 7.

The questions of whether the vesicles' sizes in the EM micrographs are consistent with secretory vesicles have not been addressed. With a scale bar of 200nm in Fig. 7e, some of the vesicles are 1µm in size and larger.

We agree that the average vesicle size appears to be larger than a typical vesicle. However, we also contend that the size of secretory vesicles is heterogenous and also that the size can vary substantially in different cell types. For example, the secretory vesicle in pancreatic acinar cells can range from 200nm to >1 µm. Given our VSVG trafficking data demonstrating that cells with reduced cortical tension (ligation of ECM in 3D) have higher levels of protein secretion, we contend that it is highly likely that the vesicles we present in our article micrographs are indeed secretory vesicles.

In the micrographs shown in Fig. 7E, adjacent to the "membrane-enclosed compartments", there appears to be another membrane, which would make it unlikely that the compartments are intracellular. Given that cryo-ET data are available, more convincing data should be presented or the data removed.

The focus of Fig. 7E is to show typical arrangements of membrane-enclosed compartments for the two experiments, not to show whether these compartments are intracellular or extracellular. When examining different views and slices in the three-dimensional reconstructions, the plasma membrane is quite evident and it is clear that these vesicles are inside the cells. Owing to the thickness of the samples, it was not possible to show both features in one slice.

It furthermore remains unclear how filled/empty vesicles were determined. The methods section specifies that "filled" compartments were those with "discernible, individual macromolecules" (which is unlikely to be possible) or those with "somewhat smoother but very dense material". The documentation for this needs to be improved, as the current description appears to be subjective.

The difference is very obvious to those trained to look at cryogenic cellular tomograms. Because of the high noise level, we agree that it is still somewhat subjective for borderline cases. For that reason, we had three experienced cryo-EM experts make independent assessments to generate the statistical analysis described in the methods section. The standard deviation for assigning vesicles 'filled' versus 'empty' is only 10.3%, comparable to the consensus observed for manual particle picking in single particle analysis, (Zhu et al. JSB 145, 2004). In these types of tomograms, individual macromolecules appear as dense globular densities that are very easy to recognize as such particularly if they are not too closely packed (at which point their densities will merge and appear like indistinct dense material).

For Fig. 7F, a p-value is given, but no error bars are shown, it is unclear how many cells/fields from how many independent experiments the bar graph in 7F was derived from.

Those are actual total numbers of vesicles. They were evaluated from 28 tomograms for each condition. The main point of the panel is to show the relative percentages of 'filled' versus 'empty' in the two conditions and whether the difference is statistically significant.

The y-axis unit is given as "number of vesicles" - per cell, per field of view, per tomogram, per unit length of plasma membrane?

This is described in detail in the methods section. We added the relevant information to the legend as well: "Membrane-enclosed compartments were independently classified by three cryo-EM experts using 28 tomograms per condition. The resulting standard deviation for assigning 'empty' versus 'filled' was 10.3% (n=3 independent expert classifications)".

My previous comment:
"2. A paragraph in the methods section is devoted to correlative light and electron microscopy (CLEM) and data is mentioned in the text, but no CLEM data are shown."
My remaining concern:
Although stated otherwise in the rebuttal, CLEM analysis is still mentioned in main text, methods, and acknowledgements, but no CLEM data are shown.

Our refer of CLEM has been removed from the manuscript text.

EMBOJ-2021-109205R

Minor:
Fig. 5G: singular "punctum" plural "puncta" (Y-axis label). The y-axis unit is given as puncta per cell (x10^3), but it appears to be a relative measurement to the untreated 3D condition, which is set to 1, since there don't seem to be 1000 puncta in the images shown.

We corrected the Y-axis label. The Y-axis unit is accurate. The representative image shown is the “Max Projection” image of confocal stacks, which only shows the brightest voxel at each xy point of the whole stack. However, the Y-unit (puncta number) is the sum of puncta of all confocal slices obtained within a cell (not just the brightest voxel of each xy point of whole stack). Thus, the number is far higher than what is shown in the Max Projection image.

2nd Revision - Editorial Decision **22nd Jun 2022**

Dear Dr Valerie Weaver,

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

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

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

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

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

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

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

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

with
Kind regards,

Daniel Klimmeck

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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.

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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;
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  - definition of error bars as s.d. or s.e.m.

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Select "Not Applicable" only when the requested information is not relevant for your study.

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New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

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

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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure legends
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	
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Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure legends

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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

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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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
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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	Materials and Methods and Data Availability sections
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	